Université de Sherbrooke

Régulation de l'épissage alternatif du gène apoptotique bcl-x

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Thèse présentée à la Faculté de médecine et des sciences de la santé

en vue de l'obtention du grade de

philosophiae doctor (Ph.D.) en microbiologie

Le 10 janvier 2008

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Your file Votre référence
ISBN: 978-0-494-64192-7
Our file Notre référence
ISBN: 978-0-494-64192-7

Résumé

Lors de la transcription de gènes, il y a production d'un pré-ARN messager qui subira plusieurs étapes de maturation pour former l'ARN messager (ARNm) pouvant coder pour une protéine. Une de ces étapes est l'épissage qui consiste à exciser des portions (appelés les introns) pour juxtaposer les autres séquences (les exons), formant ainsi l'ARNm. Lors de l'épissage alternatif, un exon peut parfois être excisé menant à la formation d'un autre isoforme d'ARNm, donc possiblement d'une autre protéine, à partir d'un seul gène. Il est maintenant estimé que plus de 97% des pré-ARNm humains multi-exoniques subissent l'épissage alternatif, permettant ainsi une augmentation considérable de la quantité de protéines codées par les 30 000 gènes humains.

Certaines des protéines produites par épissage alternatif peuvent avoir des activités très différentes, voire antagonistes. Ceci est souvent le cas dans l'apoptose, soit la mort cellulaire programmée. Le gène *bcl-x*, par exemple, peut mener à la formation de deux isoformes majoritaires. Lorsque le site d'épissage 5' proximal est utilisé, il y a formation de Bcl-x_L, codant pour une protéine ayant une activité anti-apoptotique, donc favorisant la survie de la cellule. Par contre, lorsque le site d'épissage 5' distal est utilisé, il y a formation d'un ARNm ayant perdu un exon de 189 nucléotides (nt) qui code pour Bcl-x_S, qui favorisera l'apoptose. L'épissage alternatif de ce pré-ARNm, comme les autres, est évidement bien contrôlé par des séquences présentes sur l'ARN, des facteurs protéiques liant ces séquences et des signaux cellulaires régulant l'activité de ceux-ci. Ma thèse consistait à analyser ces trois aspects et mon travail a mené à la découverte de deux protéines liant le pré-ARNm de *bcl-x*

afin de réguler son épissage, ainsi que d'une région nécessaire à la signalisation cellulaire par la protéine kinase C (PKC).

Au début, j'ai aidé à l'identification des hnRNP F et H qui lient une région riche en guanidine, nommée B2G, présente dans l'exon alternatif. Ces protéines lient cet élément et ainsi augmenterait la formation de l'isoforme Bcl-x_S. Ces résultats sont présentés en annexe.

Par la suite, j'ai identifié une région contenant deux éléments antagonistes, soit B1AC et B1u, située 10 nt en amont du site Bcl-x_S. B1AC augmente l'utilisation de ce site, tandis que B1u fait le contraire, par la liaison de hnRNP K. Cette protéine est souvent fortement exprimée dans certains types de cancer, en accord avec son activité d'inhibition de l'isoforme pro-apoptotique. Ceci est présenté dans la première partie de ma thèse.

La deuxième section de ma thèse consiste à l'identification d'une grande région nommée SB1 (361 nt) située au début de l'exon 2. De façon basale, cet élément inhibe le site Bcl-x_S. Cependant, lors du blocage de l'activité de la PKC par la staurosporine, l'inhibition est perdue entrainant ainsi une forte augmentation de cet isoforme. L'inhibition de la PKC dans les lignées cellulaires cancéreuses n'entraîne pas cet effet sur l'épissage de Bcl-x, suggérant que la voie de signalisation de PKC est déréglée ou non-couplée à la régulation du gène apoptotique Bcl-x dans les cancers.

L'avancement de la compréhension de l'épissage alternatif de gènes clés impliqués dans certaines maladies, tel que *bcl-x* dans le cancer, est primordial pour une meilleure

compréhension de celles-ci. Cette thèse présente ma participation dans l'étude de la régulation de l'épissage alternatif de *bcl-x*.

Mots-clés : épissage alternatif, maturation de l'ARN, bcl-x, hnRNP K et F/H, apoptose

Table des matières

Liste des figures

Liste des abréviations

3' ss (ou 5' ss) : site d'épissage 3' (ou 5')

ADN : acide désoxyribonucléique

ARN : acide ribonucléique

ARNm : acide ribonucléique messager

pré-ARNm : ARN messager précurseurs

BH : « Bcl-2 Homology »

CRCE : « Ceramide-Responsive RNA *Cis*-Element »

CTD : « C-terminal Domain » (domaine C-terminal)

EJC : « Exon Junction Complex » (complexe de jonction d'exons)

ESE : « Exonic Splicing Enhancer »

ESS : « Exonic Splicing Silencer »

GM-CSF : « granulocyte-macrophage colony stimulating factor »

hnRNA : « heterogeneous nuclear RNA »

hnRNP : « heterogeneous nuclear ribonucleoprotein »

IL-6 : interleukin-6

ISE : « Intronic Splicing Enhancer »

° ° °

ISS : « Intronic Splicing Silencer »

kb : kilobase

kDa : kiloDalton

NMD : « Nonsense-mediated mRNA decay »

nt : nucléotide(s)

pb : paires de bases

PKC : Protéine Kinase C

PP1 : Protéine Phosphatase-1

RNAi : « RNA interference » (interférence à l'ARN)

RRM : « RNA Recognition Motif »

RT-PCR : « Reverse Transcription - Polymerase Chain Reaction » (transcription inverse - réaction en chaîne par polymérase)

SG : « Stress Granules » (granules de stress)

snRNA : small nuclear RNA

snRNP : small nuclear RiboNucleoProtein

TNF : « Tumor Necrosis Factor »

TNFR : « Tumor Necrosis Factor Receptor »

TRAF : « TNFR-associated factors »

Introduction

L'épissage constitutif

Lors de la transcription des gènes par la polymérase II, les ARN messager précurseurs (pré-ARNm) résultants font l'objet de plusieurs modifications co-transcriptionnelles afin de former l'ARNm mature qui peut par la suite coder pour une protéine. Ces modifications peuvent être l'ajout d'une coiffe en 5', la polyadénylation en 3', ou l'épissage (de Almeida et Carmo-Fonseca, 2008).

L'épissage consiste en l'excision de parties du pré-ARNm nommées introns pour accoler les régions nommées exons. La machinerie responsable de cette étape de maturation est le splicéosome, une macromolécule composée de plus de 300 protéines (Jurica et Moore, 2003) et de cinq snRNPs, soit des ribonucléoprotéines composées de snRNAs et de leurs facteurs associés. Certains éléments du splicéosome reconnaissent sur les pré-ARNm plusieurs séquences guidant l'épissage : les sites d'épissage 5' et 3', le site de branchement et la région riche en pyrimidines (Figure 1A).

Dans la voie majeure de l'épissage, ou U2-dépendante, le site d'épissage 5' (5' ss) est reconnu par le snRNP U1. SF1/mBBP, U2AF65 et U2AF35 reconnaissent respectivement le site de branchement, la région riche en polypyrimidines ainsi que le site d'épissage 3' (3' ss). Ceci forme le complexe E. Le snRNP U2 va par la suite déplacer SF1/mBBP et se lier au site de

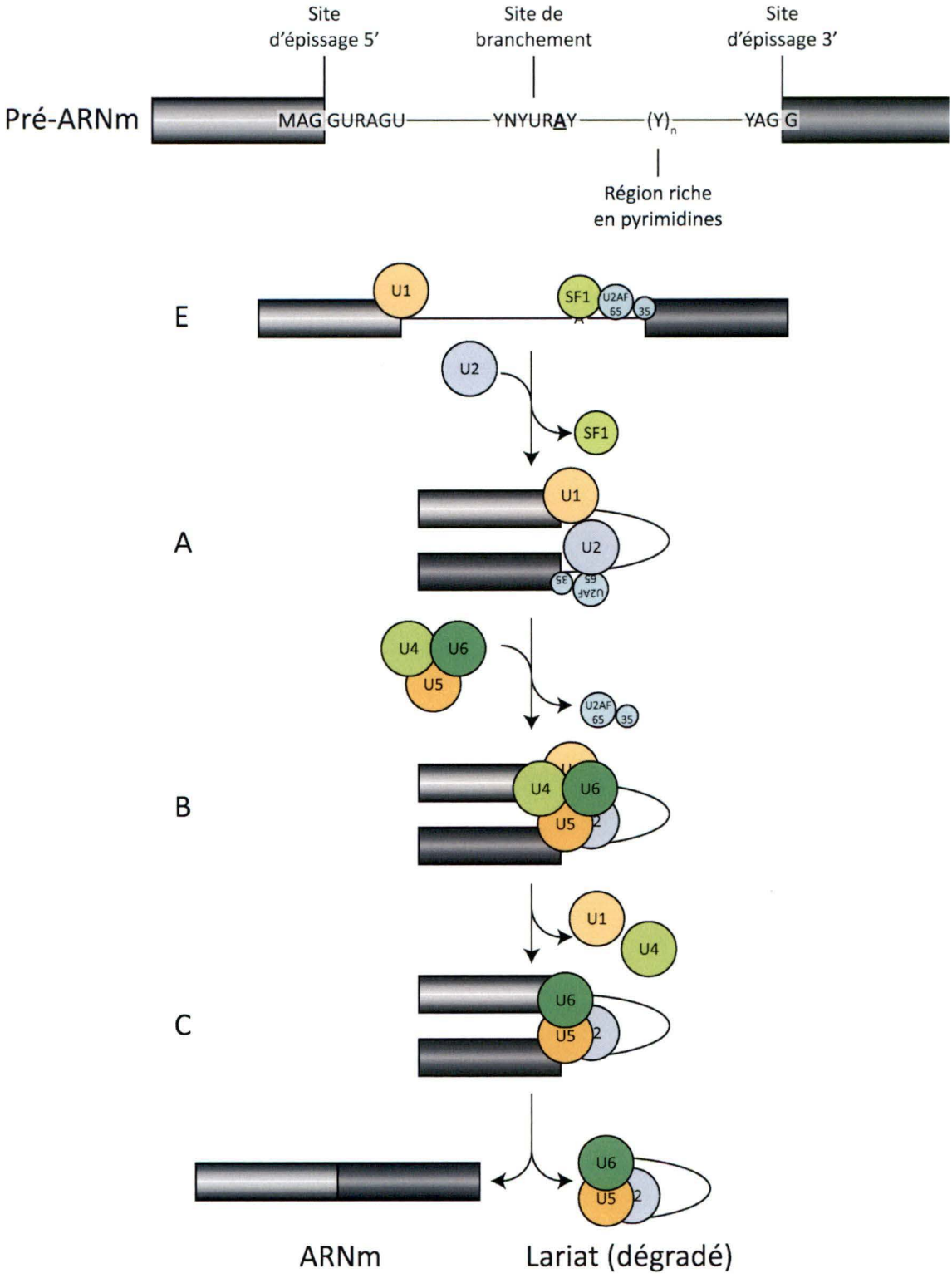

Figure 1 – L'épissage constitutif. Les exons sont représentés par des boîtes et les introns par des lignes.

branchement, aidé par son interaction avec U2AF, assemblant ainsi le complexe A (figure 1B). Par la suite, le tri-snRNP U4/U5/U6 se joint au complexe en déplaçant U2AF, induisant la formation du complexe B et un changement de conformation de l'ARN. Ceci causera la perte subséquente des snRNP U1 et U4 et la création du complexe C actif. C'est cette version finale du splicéosome qui catalysera les deux réactions de trans-estérification résultant en la juxtaposition des deux exons impliqués et l'excision de l'intron sous forme de lariat. La voie mineure de l'épissage, ou U12-dépendante, est similaire. La différence majeure réside en des sites 5' et de branchement différents ainsi que l'utilisation de quatre nouveaux snRNPs (Chang *et al.*, 2008). Les snRNPs U11, U12, U4atac et U6atac remplacent U1, U2, U4 et U6. Il n'y aurait qu'environ 700 introns de type U12 chez l'humain (Alioto, 2007; Sheth *et al.*, 2006).

L'épissage nécessite une reconnaissance précise des sites d'épissage. Un gène humain possède en moyenne 8,8 exons d'environ 145 nucléotides (nt) en moyenne, avec des introns qui font 3365 nt en moyenne (Stamm *et al.*, 2005). Certains introns peuvent même mesurer plusieurs dizaines de milliers de nucléotides et les pré-ARNm des centaines de milliers de nucléotides au total. Chez les eucaryotes supérieurs, les sites d'épissage sont souvent des séquences très dégénérées. Dans la voie majeure de l'épissage, ils varient autour d'une séquence consensus de neuf bases pour le site 5' ($(^{C}/_{A})$AG|GURAGU) et d'environ quinze pour le site 3' (YNYURAC(Y_N)YAGG). La plus haute fréquence qu'un nucléotide de ces séquences consensus s'y situe chez l'humain varie entre 35 et 80% (Sun et Chasin, 2000). Il n'y a donc que 5% de tous les sites d'épissage 5' ou 3' qui constituent la séquence consensus parfaite. De plus, de multiples pseudo-sites d'épissage existent dans les introns des pré-ARNm. Une étude effectuée sur le pré-ARNm du gène *hprt* a trouvé qu'il y avait des

centaines de sites d'épissage 5' ou 3' non-utilisés sur ce transcrit de 42 kb (Sun et Chasin, 2000). Même en tenant compte des caractéristiques classiques d'exons, ceci représente 103 « faux » exons nommés pseudo-exons. Afin de guider l'épissage vers les sites d'épissage réels, des séquences activatrices existent dans les exons (ESE) ainsi que des séquences inhibitrices (ESS).

Les ESE sont liées par des facteurs nommés protéines SR (décrites plus loin dans « Les protéines SR », page 11), qui aideront à recruter les différentes composantes du splicéosome dont le snRNP U1 et U2AF aux sites d'épissage (Eperon *et al.*, 2000; Graveley *et al.*, 2001; Wang *et al.*, 1995; Zahler et Roth, 1995). De plus, ces protéines permettent au snRNP U1 lié au site d'épissage 3' de recruter le snRNP U2 au site 3', dans un processus de définition d'exon (Boukis *et al.*, 2004; Lavigueur *et al.*, 1993). L'interaction du domaine de répétitions arginine-sérine (RS) de ces protéines avec le site d'épissage 5' et le site de branchement aiderait aussi à la formation du splicéosome (Shen et Green, 2004; Shen *et al.*, 2004). Les ESS agissent de façon contraire, soit en inhibant la formation du splicéosome par le masquage des sites d'épissage ou l'inhibition de l'interaction entre les différents facteurs du splicéosome (Amir-Ahmady *et al.*, 2005; Eperon *et al.*, 2000; House et Lynch, 2006; Sharma *et al.*, 2005). La combinaison des ESE, favorisant les sites réels, ainsi que des ESS qui inhibent les pseudo-sites d'épissage, permet au splicéosome de bien reconnaître les bonnes séquences qui sont perdus dans une mer de nucléotides.

Tel que mentionné précédemment, les étapes de l'épissage sont co-transcriptionnelles. Durant la transcription chez la levure, il a été démontré qu'il y a un assemblage séquentiel des snRNPs U1, U2 et U5 sur le pré-ARNm naissant (Gornemann *et al.*, 2005; Tardiff et

Rosbash, 2006). De plus, le snRNP U1 ainsi que des protéines SR interagissent avec l'ARN polymérase II (Das *et al.*, 2007), probablement par la queue C-terminale (CTD) de la polymérase (de Almeida et Carmo-Fonseca, 2008; de la Mata et Kornblihtt, 2006; McCracken *et al.*, 1997; Misteli et Spector, 1999). La transcription de gènes par une polymérase ne contenant pas de queue CTD, constituée de 52 répétitions de sept acides aminés (YSPTSPS) chez les mammifères (Corden, 1990), mène à un épissage peu efficace (McCracken *et al.*, 1997). Ce recrutement des facteurs d'épissage aux sites de transcription serait donc essentiel pour l'efficacité de l'épissage *in vivo*.

L'épissage alternatif

Chez les eucaryotes supérieurs, certains exons peuvent parfois être exclus lors de l'épissage, un évènement nommé épissage alternatif. Ceci mènera à plusieurs ARNm matures, soit différents isoformes, contenant des séquences différentes à partir d'un seul gène. Loin d'être l'exception, il est estimé que plus de 97% des gènes multi-exoniques sont épissés de façon alternative (Pan *et al.*, 2008). De plus, 86% de tous les gènes humains sont épissés de façon à créer un isoforme mineur dont l'abondance relative représente au moins 15% des ARNm du gène (Wang *et al.*, 2008).

Un exemple particulièrement marquant du potentiel de l'épissage alternatif est le gène *Dscam* chez la drosophile qui peut théoriquement mener à la formation de plus de 38 000 isoformes (Schmucker *et al.*, 2000). Chez l'humain, le gène *Slo* mène à plus de 500 isoformes possibles. L'épissage alternatif de son homologue chez le poulet, *cSlo*, a été démontré

comme étant relié à la fréquence captée par les cellules ciliées dans le conduit auditif. (Black, 1998; Graveley, 2001). Les trois gènes de la neurexin peuvent mener à plus de 2000 isoformes dont l'épissage alternatif régulerait la synaptogénèse et le maintien des synapses fonctionnels (Maniatis et Tasic, 2002; Rowen *et al.*, 2002). En produisant une diversité d'ARNm mature par l'inclusion ou non d'exons, l'épissage alternatif permet d'augmenter considérablement le répertoire protéique que la cellule humaine peut produire à partir d'un génome contenant seulement deux fois plus de gènes que la Drosophile et deux fois moins que l'estimation pour le riz (Adams *et al.*, 2000; Goff *et al.*, 2002; Lander *et al.*, 2001; Yu *et al.*, 2002).

Il existe plusieurs types d'épissage alternatif. Le cas le plus répandu est la présence d'exons que l'on nomme cassette (figure 2A) qui peuvent être inclus ou non dans un ARNm mature.

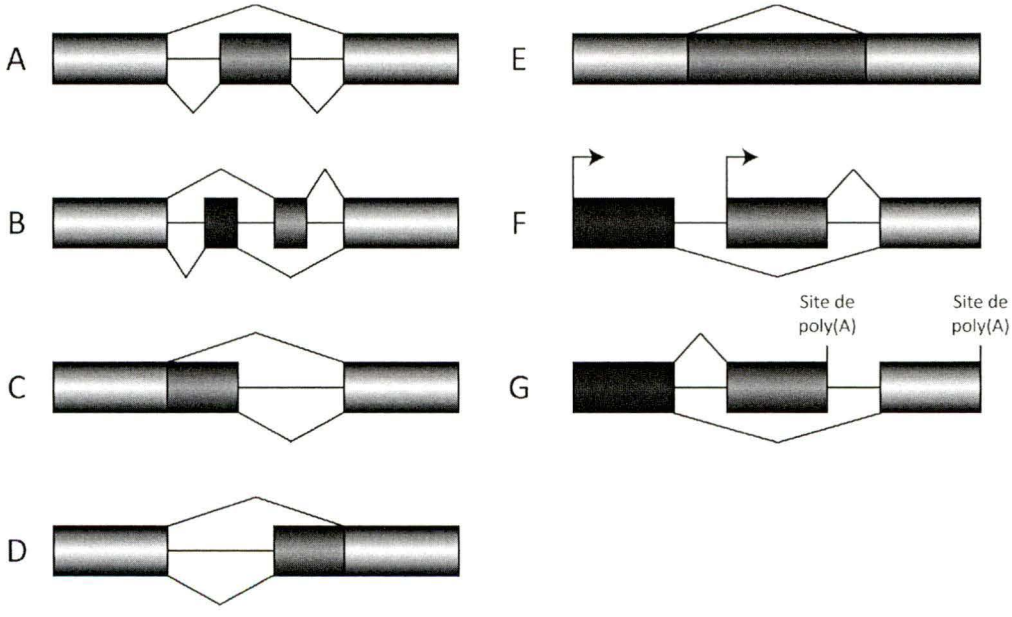

Figure 2 – Les types d'épissage alternatif. *A.* Exon cassette. *B.* Exons mutuellement exclusifs. *C.* Sites d'épissage 5' alternatifs. *D.* Sites d'épissage 3' alternatifs. *E.* Rétention d'intron. *F.* Promoteurs alternatifs. *G.* Sites de poly-adénylation alternatifs.

Occasionnellement, des exons cassettes qui se suivent peuvent être mutuellement exclusifs (figure 2B), c'est-à-dire qu'un seul sera conservé. Ceci est le cas entre autres du gène *Dscam* mentionné précédemment dans lequel il y a quatre unités d'épissage qui possèdent plusieurs exons mutuellement exclusifs menant ainsi aux 38 016 isoformes prédits. D'autres fois, il peut y avoir deux sites d'épissage 3' ou 5' en compétition (figure 2C et 2D) ou certains exons peuvent ne pas être épissés (figure 2E). Finalement, il peut y avoir plusieurs sites d'initiation de transcription menant à des premiers exons différents (figure 2F), ou encore plusieurs sites de polyadénylation (figure 2G). Le gène humain de la basonuclin 2 peut mener à près de 90 000 isoformes d'ARNm potentiels par l'utilisation de six promoteurs différents, d'au moins vingt-trois exons alternatifs et de quatre sites de poly-adénylation (Vanhoutteghem et Djian, 2007). Ceci mène à des ARNm potentiels d'une longueur variant entre 0,5 et 13 kb, dont au moins un a été démontré comme étant spécifique aux testicules. Tous ces isoformes encodent pour plus de 2000 protéines distinctes, de 43 à 1211 acides aminés.

Cette sélection alternative d'exons mène à une modification de la séquence de l'ARNm, entraînant la plupart du temps la délétion et/ou l'ajout d'une séquence protéique ainsi que, la moitié du temps, le déplacement du cadre de lecture (Clark et Thanaraj, 2002). La modification de la séquence protéique peut entraîner une activité nulle, différente, voire antagoniste du nouvel isoforme protéique.

Il arrive que l'épissage alternatif mène à la dégradation de l'ARNm par une voie nommée « Nonsense-mediated mRNA decay » (NMD) (pour une excellente revue de littérature, voir

Lareau *et al.*, 2007). Après chaque évènement d'épissage, un complexe demeure 20-24 nt en amont de la nouvelle jonction, soit le complexe de jonction d'exons (EJC) constitué de facteurs d'épissage et de traduction ainsi que de facteurs spécifiques au NMD (Figure 3A) (Le Hir et Andersen, 2008; Maquat, 2004). Lorsqu'il y a présence d'un codon d'arrêt de

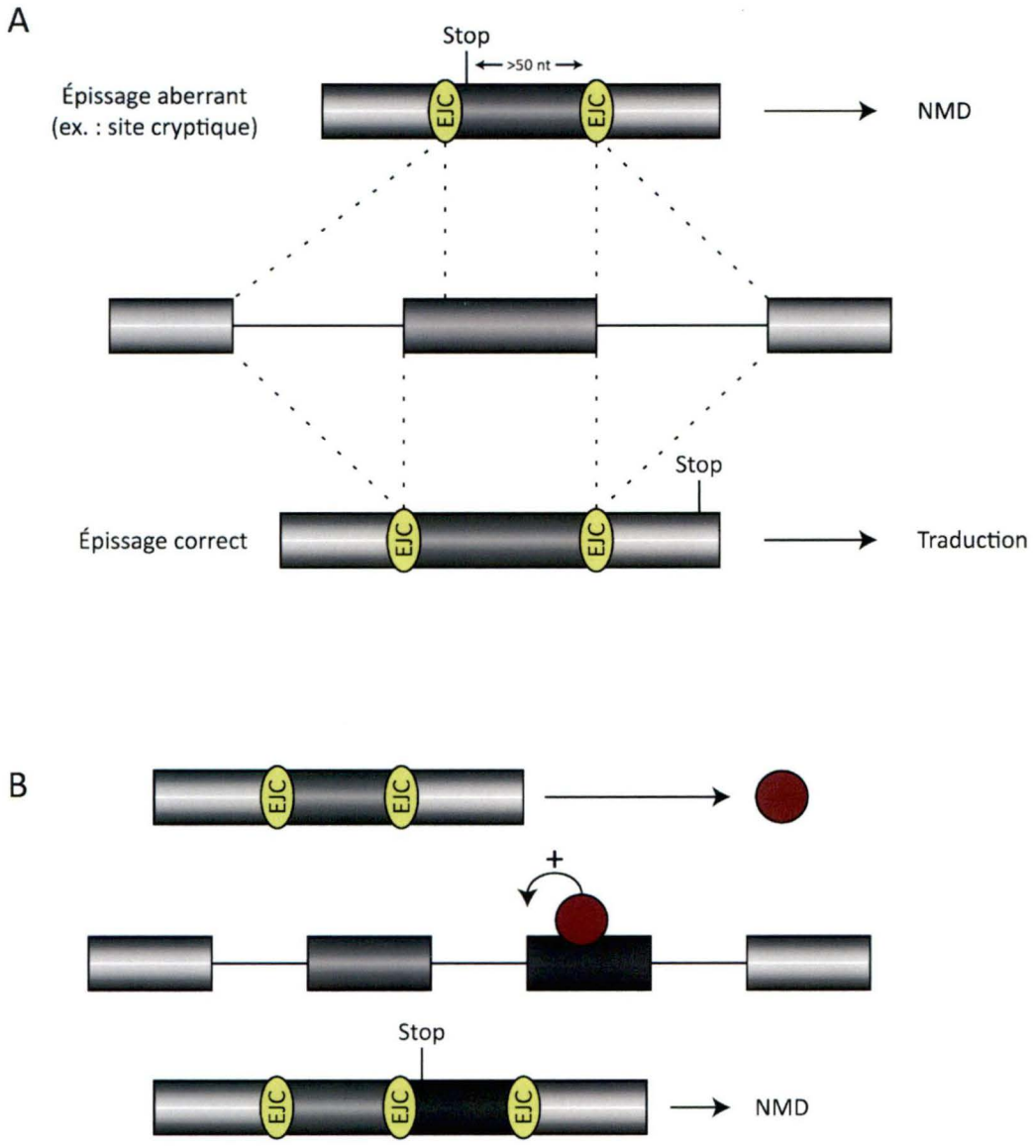

Figure 3 – La voie de dégradation médiée par le nonsense (NMD). *A.* La présence d'un codon d'arrêt de traduction à plus de 50 nucléotides (nt) du complexe de jonction d'exons (EJC) entraîne la dégradation de l'ARNm par la voie du NMD. *B.* Un facteur d'épissage peut augmenter l'inclusion d'un exon lors de l'épissage de son propre pré-ARNm, entraînant celui-ci à la dégradation par la voie du NMD. Ce mécanisme d'auto-régulation de la quantité de transcrits, et donc de protéines, est utilisé par certains facteurs d'épissage. Modifiée de Lareau *et al.*, 2007.

traduction prématuré (PTC), soit qui est situé plus de cinquante nucléotides en amont du dernier EJC, cet ARNm sera habituellement dégradé par la voie du NMD (Lejeune et Maquat, 2005). Il est estimé que plus du tiers des évènements d'épissage alternatif mène à un isoforme contenant un PTC (Green *et al.*, 2003). Cette voie permet un contrôle de qualité en éliminant une bonne portion des ARNm qui sont épissés de façon aberrante et qui pourrait mener à des protéines délétères. Elle peut aussi ajouter un nouveau niveau de contrôle des niveaux protéiques par la régulation des niveaux d'ARNm présents. Certains facteurs d'épissage contrôlent leurs niveaux de cette façon, comme les protéines SR 9G8, SRp20, ASF/SF2 et SRp55, ou les protéines hnRNP H1, I/PTB, K et M (Figure 3B) (Makeyev *et al.*, 2007; Ni *et al.*, 2007).

Plusieurs aspects peuvent agir sur la sélection des sites d'épissage. Plus un site d'épissage ressemble au site consensus, donc est fort, plus celui-ci a de chances d'être utilisé qu'un site faible qui dévie beaucoup de la séquence consensus. Les sites d'épissage alternatif sont très souvent plus faibles que les sites d'épissage constitutifs. Par contre, comme il peut y avoir plusieurs sites cryptiques non-utilisés sur un pré-ARNm (Sun et Chasin, 2000), ce n'est pas la seule variable. Ainsi, il existe des séquences qui peuvent augmenter le recrutement du splicéosome et l'inclusion de l'exon (des « enhancers ») et d'autres qui diminuent l'utilisation des sites d'épissage de différentes façons (les « silencers »). Ceux-ci peuvent être introniques (ISE : « Intronic Splicing Enhancers » ou ISS : Intronic Splicing Silencers) ou exoniques (ESE : « Exonic Splicing Enhancers » ou ESS : Exonic Splicing Silencers ») (Figure 4A).

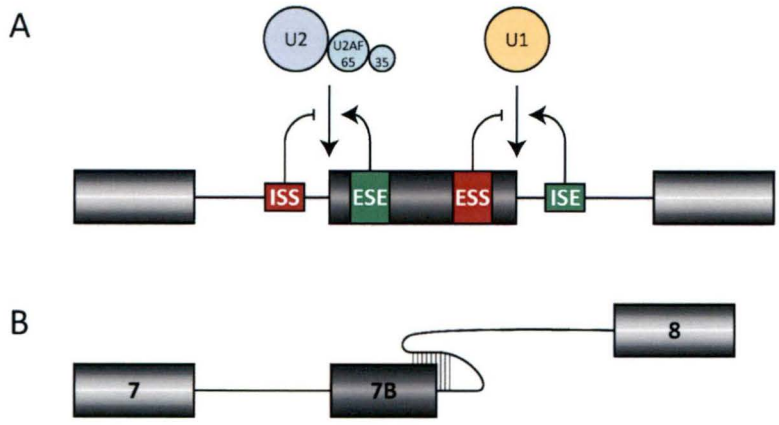

Figure 4 – Régulation de l'épissage alternatif. A. Des séquences peuvent augmenter ou inhiber la liaison du snRNP U2 ou du snRNP U1, favorisant ainsi l'inclusion ou l'exclusion, respectivement, de l'exon alternatif. B. Une structure secondaire peut masquer un site d'épissage, inhibant ainsi l'inclusion de l'exon. L'exemple illustré ici est l'élément CE6 sur le pré-ARNm de hnRNP A1.

Occasionnellement, ces éléments peuvent agir en modifiant la conformation tridimensionnelle du pré-ARNm. Par exemple, l'élément CE6, présent dans l'intron du pré-ARNm de hnRNP A1, forme un duplex avec le site d'épissage 5', ainsi qu'une partie, de l'exon 7B (Blanchette et Chabot, 1997). Ceci aura comme conséquence l'inaccessibilité de ce site pour le splicéosome et ainsi l'exclusion de l'exon alternatif 7B (Figure 4B). Une étude a aussi démontré des structures secondaires conservées d'ARN associées avec la sélection de sites d'épissage alternatif (Shepard et Hertel, 2008). La jonction de l'exon 7 et de l'intron 7 du pré-ARNm de *SMN2*, potentiellement impliqué dans l'atrophie musculaire spinale, se replie dans une structure secondaire nommée TSL2 (Singh *et al.*, 2007). Celle-ci augmente l'exclusion de l'exon 7. De plus, la structure secondaire d'un pré-ARNm peut aussi affecter la liaison de protéines régulant l'épissage alternatif de façon négative (hnRNP A1) ou positive (B52, SRp55 et NOVA-1) (Buratti et Baralle, 2004).

La plupart des séquences influençant l'épissage le font par l'entremise de la liaison d'une protéine. Les deux grandes familles de protéines modulatrices qui reconnaissent ces sites de liaison sont les protéines SR et hnRNPs. Auparavant, on accordait aux protéines SR une

activité de stimulation de l'épissage et aux protéines hnRNPs, une inhibition de l'épissage. À l'origine, ceci avait été démontré par la modification de l'épissage alternatif de la beta-tropomyosine dépendant du ratio de hnRNP A1 et de la protéine SR ASF/SF2 (Mayeda *et al.*, 1993). Cependant, cette distinction est de moins en moins actuelle, avec la découverte de protéines SR inhibant l'épissage et des hnRNPs le stimulant.

Les protéines SR

Dix protéines à l'origine, les protéines SR ont été identifiées par des caractéristiques biochimiques communes, soit l'immunoreconnaissance et la précipitation dans des concentrations élevées de magnésium. Celles-ci sont dues à la présence du domaine RS, composé de répétitions des acides aminés arginine (R) et sérine (S) qui sont généralement phosphorylés. Ce domaine serait responsable des interactions permettant le recrutement du splicéosome et possédant aussi une affinité à l'ARN (Shen et Green, 2004; Shen *et al.*, 2004). De plus, les protéines SR classiques possèdent un ou deux domaines liant l'ARN, soit les RRM (« RNA Recognition Motif »). Il existe aussi plusieurs autres facteurs d'épissage (Tableau 1) contenant un domaine RS mais contenant cependant un autre type de domaine de liaison à l'ARN, tels que U2AF65 et U2AF35 ou certaines kinases comme ClkSty-1, -2 et -3 (Lin et Fu, 2007).

Outre leur effet sur la reconnaissance des exons durant l'épissage constitutif, les protéines SR peuvent moduler l'épissage alternatif de plusieurs façons. Ces facteurs peuvent lier des séquences consensus présentes sur les pré-ARNm et augmenter la liaison du snRNP U1 au site d'épissage 5' ou U2AF au site d'épissage 3' (Figure 5A). Par exemple, ASF/SF2 peut

Classe	Facteurs	Domaines clés	Fonctions
Protéines SR classiques	SRp20	Un ou deux RRMs ainsi qu'un domaine RS	Épissage constitutif et alternatif
	ASF/SF2		
	SC35		
	9G8		
	SRp40		
	SRp55/B52		
	SRp75		
Protéines SR additionnelles	hTRA2α	Un ou deux RRMs ainsi qu'un domaine RS	Régulation positive et négative de l'épissage
	hTRA2β		
	RNPS1		
	SRp38		
	SRp30c		
	P54		
	SRrp35		
	SRrp53		
	SRp86		
Protéines liant l'ARN, reliées aux protéines SR	U2AF65	RRM, domaine PWI, doigt zinc et un domaine RS	Facteurs d'épissage ou co-activateurs
	U2AF35		
	Urp		
	HCC1/CAPER		
	U1-70K		
	hSWAP		
	Pinin		
	Sip1		
	SR-A1		
	ZNF265		
	SRm160		
	SRm300		
Enzymes et régulateurs contenant un domaine RS	hPRP5	Boîte DEAH, domaines kinase, domaine peptidyl-prolyl isomérase	Réarrangement du splicéosome et modification de facteurs d'épissage
	hPRP16		
	Prp22/HRH1		
	U5-100K/hPRP28		
	ClkSty-1		
	ClkSty-2		
	ClkSty-3		
	CLASP		
	Prp4K		
	CrkRS/CRK7/CDK12		
	CDC2L5		
	CCNL1		
	CCNL2		
	SR-cyp		

Tableau 1 – Les protéines SR. Un nouveau type de classement des protéines SR par domaines clés et fonctions dans l'épissage. Modifié de Lin et Fu, 2007.

augmenter la liaison du snRNP U1 à deux sites d'épissage 5' en compétition, favorisant l'utilisation du site proximal (Eperon *et al.*, 2000). Cette même protéine peut augmenter l'inclusion d'un exon alternatif dans le pré-ARNm de *bGH*.

Occasionnellement, les protéines SR liées près d'un site d'épissage peuvent réduire son utilisation (Figure 5B). SRp30c peut lier l'élément CE9 du pré-ARNm de hnRNP A1 et sa liaison inhibe l'utilisation du site 3' en aval (Simard et Chabot, 2002). ASF/SF2 peut inhiber l'épissage du pré-ARNm tat de HIV-1 *in vivo* (Wang *et al.*, 1998) ainsi que favoriser l'exclusion de l'exon 11 du gène *Ron*, formant l'isoforme ΔRon qui possède une activité constitutive sur la motilité et la prolifération cellulaire (Ghigna *et al.*, 2005). SRrp86 peut moduler l'activité de plusieurs protéines SR, activant SRp20 et inhibant SC35, mais est à son tour inhibiée par plusieurs facteurs d'épissage (Barnard *et al.*, 2002; Li *et al.*, 2003). SRp38 (ou SRrp40) devient déphosphorylée et agit comme inhibiteur général d'épissage durant la mitose ou lors d'une exposition à la chaleur (Shin *et al.*, 2004; Shin *et al.*, 2005).

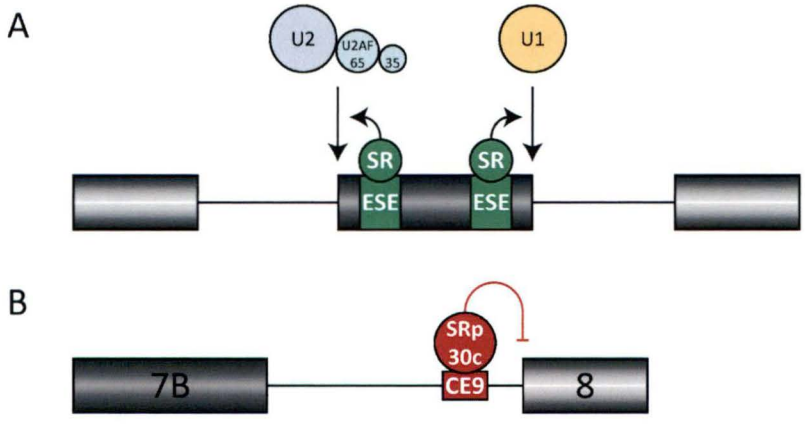

Figure 5 – Mécanismes d'action des protéines SR. *A.* Les protéines SR peuvent se lier à des éléments dans les exons et augmenter l'inclusion d'un exon. *B.* Les protéines SR peuvent aussi inhiber l'utilisation d'un site d'épissage. Ici, SRp30c lie CE9 sur le pré-ARNm de *hnRNP A1* et inhibe l'utilisation du site d'épissage 3' à proximité.

Outre leurs rôles sur l'épissage, les protéines SR peuvent agir sur plusieurs autres processus impliquant les ARNm (Huang et Steitz, 2005). ASF/SF2 peut activer la traduction en activant la phosphorylation de 4E-BP1, diminuant ainsi l'activité de cet inhibiteur de traduction cap-dépendent (Michlewski *et al.*, 2008). De plus, les protéines SR peuvent agir sur l'exportation des ARNm du noyau vers le cytoplasme. 9G8 et SRp20 peuvent lier un élément de l'ARNm de l'histone *H2a*, ne contenant pas d'intron, et ainsi favoriser sa sortie du noyau en s'associant avec TAP/NXF1, un récepteur d'exportation d'ARNm (Huang *et al.*, 2003; Huang et Steitz, 2001). Quelques protéines SR, dont surtout ASF/SF2, favorisent la dégradation d'un ARNm modèle (Zhang et Krainer, 2004). La déplétion d'ASF/SF2 par RNAi augmente aussi la demi-vie de l'ARNm de PKCI-r de plus de 6 fois. (Lemaire *et al.*, 2002). Finalement, la perte de la protéine ASF/SF2 induit l'apoptose dans une lignée cellulaire de poulet malgré la diminution de l'isoforme ICAD-L, nécessaire pour le bon repliement de la nucléase dégradant l'ADN, ainsi que l'effet positif de ASF/SF2 sur la formation de l'isoforme pro-apoptotique Casp-2L (voir « L'apoptose et l'épissage alternatif », en page 28) (Jiang *et al.*, 1998; Li *et al.*, 2005).

Les protéines hnRNPs

Historiquement, les protéines hnRNPs (« heterogeneous nuclear ribonucleoprotein ») étaient un groupe de protéines repêchées par leur association avec des transcrits primaires de haut poids moléculaire (hnRNA : « heterogeneous nuclear RNA », ou le pré-ARNm) (Dreyfuss *et al.*, 1993). À ce jour, environ 24 hnRNPs ont été identifiées possédant des similarités de séquences et des assemblages de domaines caractéristiques. Celles-ci se lient à des sites consensus relativement dégénérés (Tableau 2).

hnRNPs	Sites de liaison
A1/A2	UAGRG$^{A}/_{U}$
	UAGG
C	U rich
	AGUAUUUUUGUGGA
D	AU rich
	AUUUA
	UUAG
E	poly C
F/H/H'/2H9	GGGA (tous)
	GGGC (H et H')
G	AAGT
I (PTB)	UCUU(C)
	CUCUCU
	Y_6CUUCUCUCUY_6
K	poly C
	AUC$_{3/4}$($^{U}/_{A}$)($^{A}/_{U}$)
	CCAUCN$_{2-7}$(A/U)CCC(A/U)N$_{7-18}$UCA(C/U)C
L	CA rich
M	poly G & poly U
	purine riche (Hrp59)
P2	GGUG
Q	U and AU rich
R	poly U
U	poly G
	poly A

Tableau 2 – Les protéines hnRNPs. Quelques protéines hnRNPs ainsi que leurs sites de liaison respectifs. N : tous les nucléotides; R : G ou A; Y : U ou C. Modifié de Martinez-Contreras *et al.*, 2007.

Auparavant, un rôle de chacune de ces protéines hnRNPs dans l'épissage alternatif n'étaient pas démontré pour toutes. Cependant, l'implication de ces protéines sur l'épissage alternatif a longtemps été étudiée en cas par cas, avec des gènes modèles. Une étude récente ciblait quatorze hnRNPs (A1, A2, C, D, DL, F, G/RBM-X, H, I/PTB, K, L, M, Q/Syncrip et R) par interférence à l'ARN tout en vérifiant par RT-PCR 56 événements d'épissage alternatif de gènes apoptotiques dans trois lignées cellulaires (Venables *et al.*, 2008b). Chacun de ces hnRNPs influence les ratios d'épissage. Les effets variaient entre un événement d'épissage alternatif affecté par hnRNP M, à 24 pour hnRNP K. De plus, cette régulation de l'épissage alternatif semble dépendre des types cellulaires puisque seulement cinq des 24 cibles de

hnRNP K étaient communes aux trois lignées cellulaires, et dix étaient uniquement dans une lignée.

L'effet majoritairement connue des protéines hnRNP sur l'épissage alternatif est l'exclusion d'exons (Figure 6A). hnRNP A1 peut inhiber l'utilisation de sites d'épissage lorsque liée à un site intronique ou exonique (Asai *et al.*, 2003; Expert-Bezancon *et al.*, 2004; Guil *et al.*, 2003; Hallay *et al.*, 2006; Hua *et al.*, 2008; Kashima *et al.*, 2007a; Kashima *et al.*, 2007b; Singh *et al.*, 2004a; Zhao *et al.*, 2004). Cet effet est entre autres aussi observé pour hnRNP F/H (Caputi et Zahler, 2002; Chen *et al.*, 1999; Coles *et al.*, 2008; Masuda *et al.*, 2008; Sironi *et al.*, 2004), hnRNP I/PTB (Carstens *et al.*, 2000; Zuccato *et al.*, 2004) et hnRNP L (Melton *et al.*, 2007; Rothrock *et al.*, 2005).

La liaison des hnRNP à ces séquences peut avoir plusieurs actions. Une première action possible est l'occlusion d'un site de liaison pour une protéine SR. Dans l'exon 2 du gène *tat* de HIV-1, hnRNP A1 se lie à l'élément ESS2 et ainsi inhibe la reconnaissance d'ESE2 par SC35 (Figure 6A) (Zahler *et al.*, 2004). Ceci diminue donc l'utilisation du site 3' à proximité. Dans l'exon 3 du même gène, hnRNP A1 peut aussi agir par un phénomène de nucléation. Sa liaison au site de haute affinité dans l'ESS3 entraînera l'attachement d'autres hnRNP A1 en aval sur l'ARN ce qui masquera le site 3' (Mayeda et Krainer, 1992). La liaison d'ASF/SF2 à l'ESE3 bloquera cette propagation. Un autre mécanisme d'action connu pour hnRNP A1, F/H et possiblement I/PTB est la formation de boucles dans l'ARN (« looping-out ») (Chabot *et al.*, 1997; Chou *et al.*, 2000; Martinez-Contreras *et al.*, 2006; Nasim *et al.*, 2002). Ceci a lieu quand ces protéines se lient de part et d'autre d'un exon. L'interaction entre les deux protéines va rapprocher les sites d'épissage éloignés et ainsi favoriser l'exclusion de l'exon

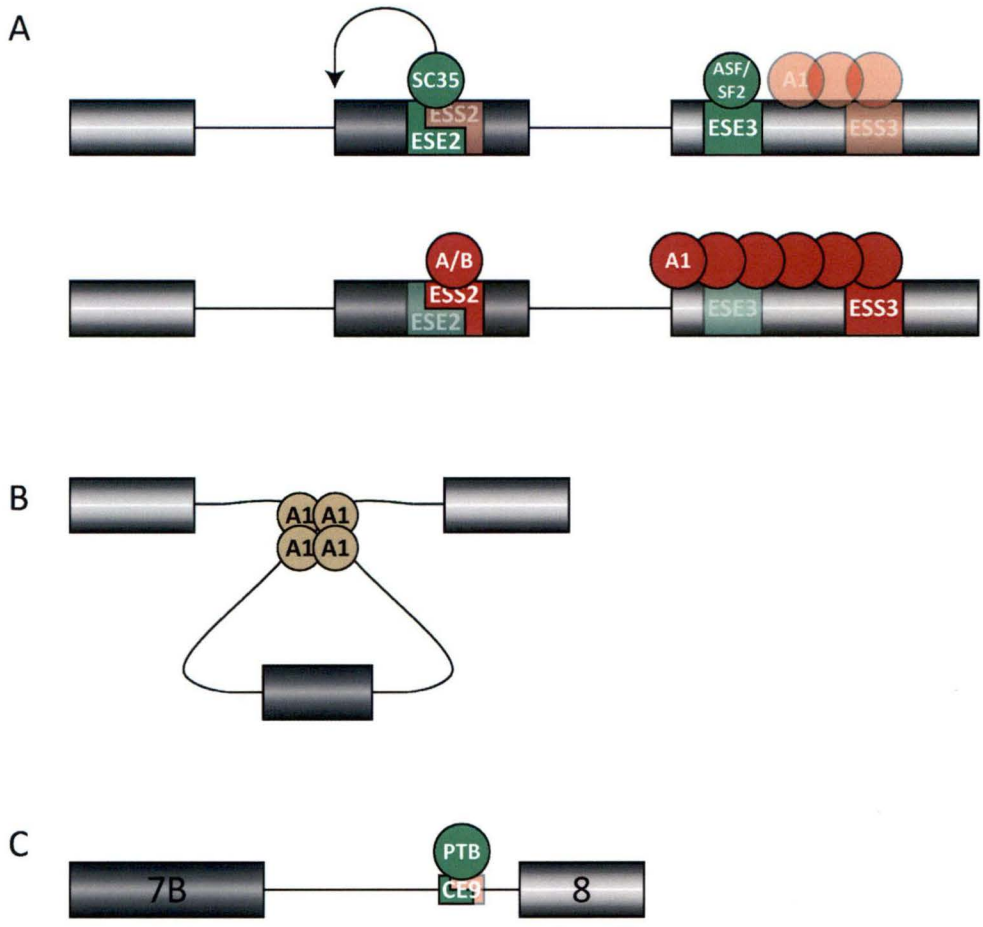

Figure 6 – Mécanismes d'action des protéines hnRNPs. A. Dans l'exon 2 du gène *tat* de HIV-1, la liaison de hnRNP A1 à l'ESS2 empêche la liaison de SC35, entraînant l'exclusion de cet exon. Dans l'exon 3, hnRNP A1 se lie à un site de haute affinité dans l'ESS3 et pourrait se propager sur l'ARNm en bloquer le site 5' de cet exon. La liaison de la protéine SR ASF/SF2 à l'ESE3 inhiberait cette nucléation.

central. Ceci pourrait aussi aider à augmenter l'efficacité de l'épissage de longs introns en rapprochant les sites d'épissage.

HnRNP I, ou PTB, est bien reconnue pour son activité d'inhibition des sites d'épissage à proximité par l'empêchement de la définition de l'intron ou de l'exon (Chan et Black, 1997; Cote *et al.*, 2001b; Sharma *et al.*, 2005; Wagner *et al.*, 2005). Cependant, les protéines hnRNPs n'agissent pas qu'en favorisant l'exclusion d'exons. Plusieurs hnRNPs possèdent même plutôt une activité connue majoritairement d'inclusion d'exons, comme hnRNP F/H, L

et Q (Venables *et al.*, 2008b). Le ciblage de hnRNP C par interférence à l'ARN démontre un effet de cette protéine seulement sur l'augmentation de l'inclusion dans 15 des 53 événements d'épissage alternatifs vérifiés. Lorsque PTB se lie à une séquence située dans l'élément CE9 du pré-ARNm de hnRNP A1, il enlève l'inhibition causée par SRp30c en la déplaçant de son site de liaison dans ce même élément (comparer Figure 6C et figure 5B). Plusieurs autres hnRNPs peuvent agir de façon positive ou négative sur l'inclusion de différents exons. hnRNP G/RBM-X promeut l'inclusion de l'exon 8 de Tau, mais aussi l'exclusion de l'exon 10 dans ce même pré-ARNm (Venables *et al.*, 2008b; Wang *et al.*, 2004). hnRNP F/H, K, L et R possèdent aussi cette dualité d'activité. On peut donc se permettre d'affirmer que la famille des hnRNPs ne devrait plus être uniquement considérée comme favorisant l'exclusion d'exons.

Tout comme les protéines SR, les protéines hnRNPs n'agissent pas que sur l'épissage alternatif. hnRNP A1 peut aussi agir sur la biogénèse des télomères et le transport des ARNm. hnRNP K régule la traduction, la stabilité des ARNm et la transcription. Il y aussi des rôles de diverses hnRNPs sur ces différents aspects ainsi que la poly-adénylation (voir la revue de littérature : Krecic et Swanson, 1999).

La transcription et l'épissage alternatif

L'épissage étant un processus co-transcriptionnel de maturation du pré-ARNm, la transcription peut aussi moduler l'épissage alternatif. Plusieurs modèles non exclusifs existent. L'identité du promoteur peut influencer plusieurs facteurs. La vitesse de la transcription de la polymérase II dépendant du promoteur peut favoriser l'inclusion ou

l'exclusion d'un exon. Une transcription plus rapide peut ne pas permettre la reconnaissance des sites d'épissage et favorisera ainsi l'exclusion d'un exon. C'est le cas d'EDI, un exon alternatif dans le pré-ARNm de la fibronectine. Celui-ci possède un site d'épissage 3' moins fort que l'exon situé en aval. Lorsque la transcription est rapide, les deux sites sont disponibles en peu de temps, favorisant la sélection du site d'épissage 3' fort et ainsi l'exclusion de l'exon central (Figure 7). Cependant en ralentissant la transcription, ou en ajoutant des sites de pause dans l'exon central, on permet au splicéosome de se former sur les sites d'épissage du premier intron et ainsi d'inclure l'exon alternatif (de la Mata *et al.*, 2003; Kadener *et al.*, 2001; Kadener *et al.*, 2002; Nogues *et al.*, 2002).

Le promoteur peut aussi influencer la phosphorylation de la queue CTD (« C-terminal domain ») de la polymérase II, formant la phospho-CTD. Durant la transcription, les patrons de phosphorylation sont activement modifiés et il est pensé que ceci pourrait moduler la liaison de facteurs à la CTD (Phatnani et Greenleaf, 2006). Plusieurs facteurs d'épissage possèdent une affinité pour la queue CTD dans son état phosphorylé (Kim *et al.*, 1997; Morris et Greenleaf, 2000; Mortillaro *et al.*, 1996; Yuryev *et al.*, 1996). Une étude à montré que certaines protéines SR, dont 9G8 et ASF/SF2, étaient recrutées par la phospho-CTD (Das *et al.*, 2007). SRp20 peut aussi, par son interaction avec la CTD influencer l'épissage alternatif de certains pré-ARNm (de la Mata et Kornblihtt, 2006). De plus, la protéine SR SC35 est nécessaire pour la transcription efficace de certains gènes (Lin *et al.*, 2008). La transcription peut donc influencer l'épissage alternatif directement par sa vitesse mais aussi par un recrutement différentiel de facteurs d'épissage sur le pré-ARNm naissant.

Les voies de signalisation

Un grand intérêt de l'épissage alternatif est la possibilité de modifier la protéine produite par un gène rapidement en réponse à un signal intracellulaire ou extracellulaire à travers des voies de transduction des signaux.

Modifications post-traductionnelles des facteurs d'épissage

Ces voies de signalisation peuvent moduler l'activité des facteurs d'épissage par des modifications post-traductionnelles comme la phosphorylation du domaine arginine/sérine (RS) des protéines SR. Ceci pourra influencer la liaison de ces protéines, leur action sur l'épissage, leur localisation ou leur stabilité. Une kinase impliquée dans la phosphorylation des facteurs d'épissage est Clk/Sty. Une hypo- ou hyperphosphorylation par celle-ci des protéines SR inhibe l'épissage constitutif et modifie l'épissage alternatif (Prasad *et al.*, 1999).

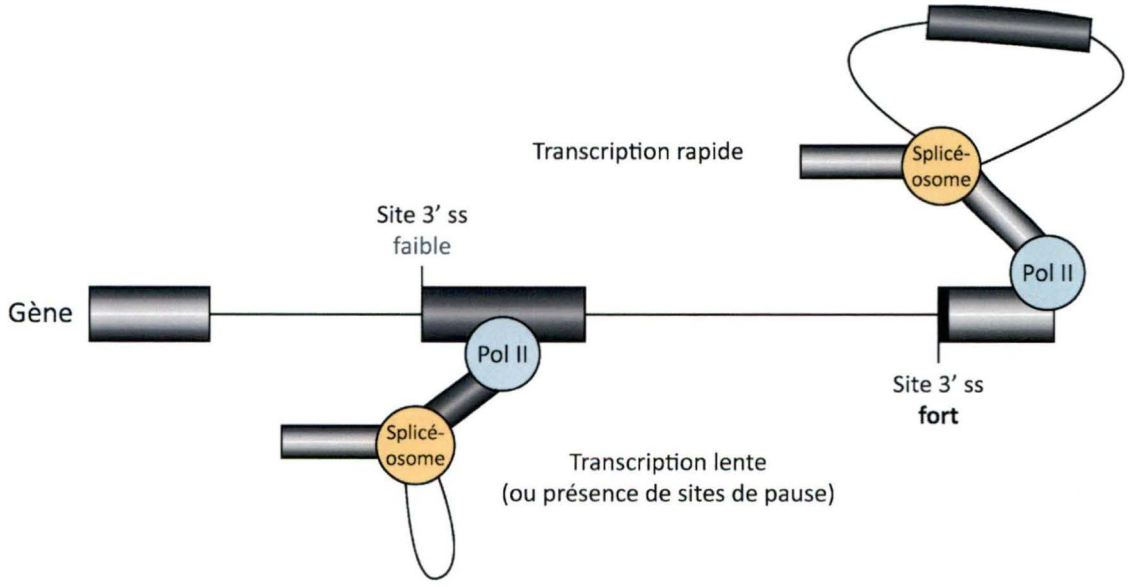

Figure 7– L'influence de la transcription sur l'épissage alternatif. Une transcription rapide entraîne l'apparition des sites d'épissage en un laps de temps ne permettant pas au splicéosome d'enlever le premier intron. La force des sites déterminera lequel sera utilisé. Lors d'une transcription lente, ou en présence de sites de pause de transcription, le splicéosome peut se former et enlever le premier intron, favorisant l'inclusion de l'exon central.

L'hyperphosphorylation de SRp55 causée par la surexpression de Clk/Sty entraînera sa dégradation par le protéasome (Lai *et al.*, 2003). L'activité de la protéine SRp38 est extrêmement régulée par son état de phosphorylation. Tel que mentionné précédemment, SRp38 agit comme répresseur général d'épissage lorsque son domaine RS est déphosphorylé par la phosphatase PP1 durant la mitose ou suite à un traitement choc à la chaleur (Shi et Manley, 2007; Shin *et al.*, 2004; Shin *et al.*, 2005). Par contre, la phosphorylation de cette protéine modifierait l'épissage alternatif du pré-ARNm du récepteur B de glutamate en présence d'un cofacteur indéterminé (Feng *et al.*, 2008). La phosphatase PP1, ainsi que PP2A, peut agir sur plusieurs autres protéines SR ainsi que des composantes des snRNPs U2 et U5 (Mermoud *et al.*, 1992; Misteli et Spector, 1996; Shi *et al.*, 2006). PP1 médie aussi l'effet de la céramide sur l'épissage des pré-ARNm de la caspase-2 et de bcl-x (Chalfant *et al.*, 2002). La phosphorylation des protéines SR peut aussi agir sur leur localisation cellulaire et ainsi possiblement leur activité sur l'épissage (Lin *et al.*, 2005).

Les protéines hnRNPs sont également régulées par la phosphorylation. hnRNP A1 peut être phosphorylé par la kinase Mnk1/2 de la voie de signalisation MAPKK(3/6)-p38 dans des cellules NIH 3T3 soumises à un choc osmotique. Ceci entraîne son accumulation dans le cytoplasme dans des granules de stress (SG) et une modification de l'épissage alternatif de certains pré-ARNm (Allemand *et al.*, 2005; Guil *et al.*, 2006). L'état de phosphorylation de PTB/hnRNP I influence aussi sa localisation. Lorsque phosphorylée, cette protéine demeure dans le noyau et n'est plus retrouvée dans le cytoplasme tandis qu'une hyperphosphorylation a l'effet contraire, soit une accumulation dans le cytoplasme (Xie *et al.*, 2003). Connaissant l'activité de cette protéine, nous pouvons supposer que ceci aurait

une influence sur l'épissage alternatif. Les protéines hnRNPs peuvent aussi être modifiés par SUMOylation, méthylation ou acétylation (Chen *et al.*, 2008; Iwasaki, 2008; Kim *et al.*, 2008; Kim *et al.*, 2006; Li *et al.*, 2004; Liu et Dreyfuss, 1995; Ostareck-Lederer *et al.*, 2006; Pawlak *et al.*, 2002; Shen *et al.*, 1998; Wada *et al.*, 2002).

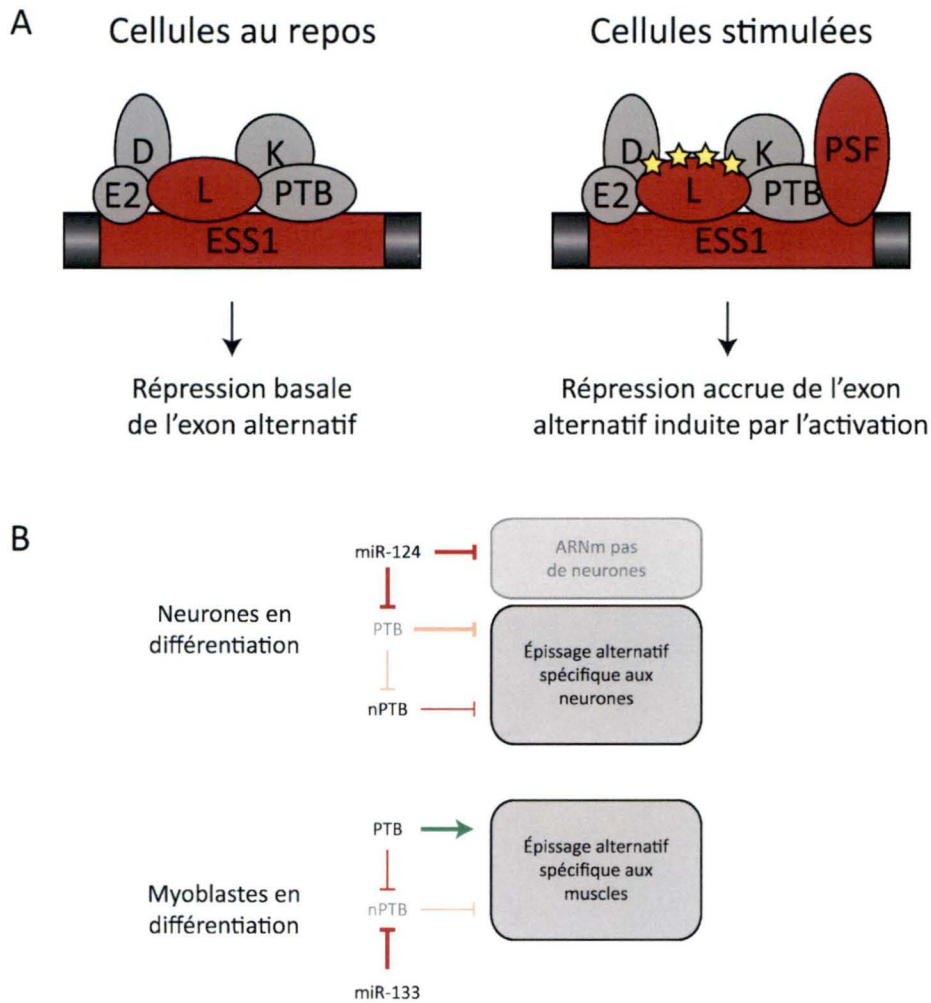

Figure 8 – La régulation de l'épissage alternatif par la signalisation cellulaire. *A.* Dans les cellules T au repos, certaines hnRNPs se lient à l'ESS1 pour exercer une répression basale faible sur l'inclusion de l'exon v4 de CD45. Lors de l'activation de ces cellules, il y a phosphorylation de hnRNP L et liaison de PSF pour une répression accrue. Modifié de Melton *et al.*, 2007. *B.* L'effet des micro-ARNs sur l'épissage alternatif. L'épaisseur des traits indiquent la force de l'action. Le micro-ARN miR-124 est exprimé dans les neurones en différentiation pour inhiber la synthèse de PTB. Ceci enlève la répression exercée par PTB sur l'inclusion d'exons spécifiques aux neurones et sur la synthèse de son homologue nPTB. nPTB pourra moduler un sous-groupe d'exons alternatifs. Dans les myoblastes en différentiation, miR-133 réprime l'expression de nPTB. Modifiée de Makeyev *et al.*, 2007.

La modification post-traductionnelle des facteurs d'épissage est sous le contrôle de différents signaux. Un modèle bien étudié est l'activation des cellules T par un antigène ou des esters de phorbol. Ceci influencera l'épissage alternatif de deux pré-ARNm encodant pour des glycoprotéines d'adhésion cellulaire : CD44 et CD45. Ce dernier code pour une tyrosine phosphate jouant un rôle dans l'activation par les récepteurs de cellules T (TCR) (Holmes, 2006). La voie de signalisation Ras ou PKC module l'inclusion de l'exon v4 par la phosphorylation de hnRNP L et la liaison de PSF à ESS1 (Figure 8A) (Lynch et Weiss, 2000; Melton *et al.*, 2007; Rothrock *et al.*, 2003; Tong *et al.*, 2005). CD44 est quant à lui impliqué dans la croissance, la survie, la différentiation et la motilité des cellules (Ponta *et al.*, 2003). Des dizaines d'isoformes ont été détectés à ce jour sur une possibilité de plus de 800, dont certains sont présents seulement dans des cancers (Naor *et al.*, 2008; Ponta *et al.*, 2003). L'épissage alternatif d'un des dix exons alternatifs, v5, est modulé par la cascade de signalisation Ras-Raf-MEK-ERK dans les cellules T activées agissant sur un élément exonique (Konig *et al.*, 1998; Weg-Remers *et al.*, 2001). Ceci semble être dépendant du coactivateur d'épissage SRm160 ainsi que de Sam68 (Cheng et Sharp, 2006; Matter *et al.*, 2002). Plusieurs autres protéines influencent l'épissage de cet exon, soit Tra-2β et YB-1 (Stickeler *et al.*, 2001; Watermann *et al.*, 2006). Ras activerait aussi l'inclusion de l'exon v6, formant ainsi l'isoforme protéique CD44v6 qui activerait à son tour Ras dans une boucle de rétroaction positive (Cheng *et al.*, 2006).

Récemment, un nouveau mécanisme de contrôle du ratio de hnRNP I/PTB et son homologue nPTB par les micro-ARNs a été découvert (Figure 8B). Ces deux protéines agissent régulièrement de façon antagoniste. Les patrons d'épissage spécifiques aux muscles et aux

neurones sont souvent régulés par hnRNP I/PTB (Li *et al.*, 2007). Lors de la différentiation cellulaire des neurones, les concentrations de PTB diminuent. Ceci va permettre la formation d'un isoforme de nPTB non-dégradé par le NMD, et ainsi une augmentation de la quantité de cette protéine. Cette baisse de PTB serait due à l'expression tissus-spécifique d'un micro-ARN miR-124 et ceci favoriserait la formation d'isoformes spécifiques aux neurones (Makeyev *et al.*, 2007). Durant le développement musculaire cependant, il y a expression du micro-ARN miR-133 dans les myoblastes en différentiation. Ceci inhibera la production de nPTB et ainsi une augmentation de l'effet de PTB sur l'épissage alternatif d'exons spécifiques aux muscles (Boutz *et al.*, 2007). Il sera intéressant de voir si l'activité de d'autres facteurs d'épissage est régulée par ces micro-ARN.

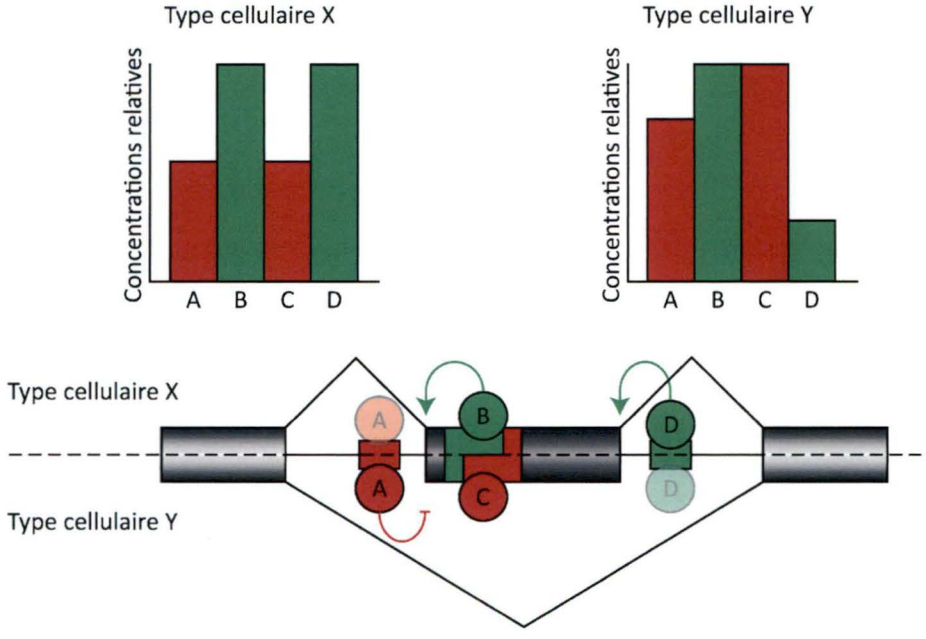

Figure 9 – Le contrôle de la spécificité dans l'épissage alternatif. Les graphiques représentent les concentrations relatives de ces facteurs d'épissage. Dans le type cellulaire X, la présence des facteurs B et D augmentent l'inclusion de l'exon alternatif. Cependant, dans le type cellulaire Y, le facteur C, possédant une plus forte affinité pour ce site, vient déplacer B. La présence additionnelle de A et l'absence de D favorise aussi davantage l'exclusion de l'exon. Modifiée de Revil *et al.*, 2006.

La spécificité de la régulation de l'épissage alternatif

Étant donné la quantité d'événements distincts d'épissage alternatif et malgré la découverte constante de nouvelles protéines ayant un rôle dans la sélection des sites d'épissage, il ne peut évidemment y avoir une protéine spécifique à chaque événement. Dans une cellule, la modification de l'épissage alternatif d'un pré-ARNm ne devrait pas obligatoirement avoir un effet global. Afin de contrer ceci, la sélection d'un site d'épissage est rarement sous le contrôle d'un seul élément et facteur (voir les revues de littérature : Fisette *et al.*, 2008; Revil *et al.*, 2007). De plus, plusieurs protéines peuvent être redondantes, comme certaines protéines SR (Lin et Fu, 2007). 9G8, ASF/SF2 et SRp20 augmentent tous l'inclusion de l'exon v9 dans le pré-ARNm (Galiana-Arnoux *et al.*, 2003). Quelques facteurs ne sont présents que dans un type de tissus, tels Nova2 et nPTB qui sont neuro-spécifiques, permettant une modulation de l'épissage dans le but de former des isoformes tissus-spécifiques (Li *et al.*, 2007; Makeyev *et al.*, 2007; Ule *et al.*, 2006). La localisation du site de liaison de certains facteurs par rapport aux sites d'épissage peut aussi moduler l'activité de ces protéines. Fox2 peut agir en tant que répresseur lorsque liée en amont d'un exon (Zhou *et al.*, 2007) ou, lorsque liée en aval, comme activateur (Ponthier *et al.*, 2006).

L'apoptose

L'apoptose est un processus induisant la mort de la cellule qui est constitué de plusieurs groupes de protéines agissant par deux voies principales (Figure 9, modifiée de Schwerk et Schulze-Osthoff, 2005). La voie extrinsèque consiste en la transmission d'un signal par un

ligand, dont la famille des TNF (« Tumor necrosis factor ») ou FasL, qui se lie à type de récepteurs nommé « récepteurs de mort ». Ces récepteurs, par l'entremise d'un domaine de mort intracellulaire, transmettent le signal apoptotique à des protéines adaptatrices qui activeront ensuite les caspases. Les caspases sont des protéases responsables du clivage des nombreux substrats qui caractérisent l'apoptose. Deux grandes familles existent, soit les caspases initiatrices qui activeront les caspases effectrices. Ces protéines existent dans la cellule sous forme inactives (pro-caspases) et c'est leur clivage qui les rendra actives (Adams, 2003).

Les caspases sont aussi communes à l'autre voie d'apoptose, soit intrinsèque. Cette voie est sensible entre autres aux dommages à l'ADN et aux agents cytotoxiques. Elle est caractérisée par le rôle central de la mitochondrie et de l'effet des protéines de la famille Bcl-2 sur le potentiel de la membrane de celle-ci (Cory et Adams, 2002; Wong et Puthalakath, 2008). Il existe plusieurs membres anti-apoptotiques (Bcl-2, Bcl-x_L, Mcl-1, Bcl-w, etc.) et pro-apoptotiques (Bim, Bak, Bax, Bcl-G, Bcl-x_S, etc.) dans cette famille (Yip et Reed, 2008). Ceux-ci possèdent tous au moins un des quatre domaines d'homologie à Bcl-2 : BH1, BH2, BH3 ou BH4 (BH : « Bcl-2 Homology »). Les membres anti-apoptotiques contiennent, pour la plupart, au moins BH1, BH2 et BH3, ainsi qu'un domaine transmembranaire (TM) permettant une localisation à la mitochondrie. Seul le domaine BH3 est nécessaire pour la dimérisation et l'activité des membres pro-apoptotiques (van Delft et Huang, 2006). La balance entre les nombreux membres antagonistes de la famille Bcl-2 est un élément clé dans la survie d'une cellule (Adams, 2003). Ces protéines sont très importantes dans le contrôle de la sortie du cytochrome c. En effet, lorsque celui-ci sort de la mitochondrie, il s'associe à la protéine

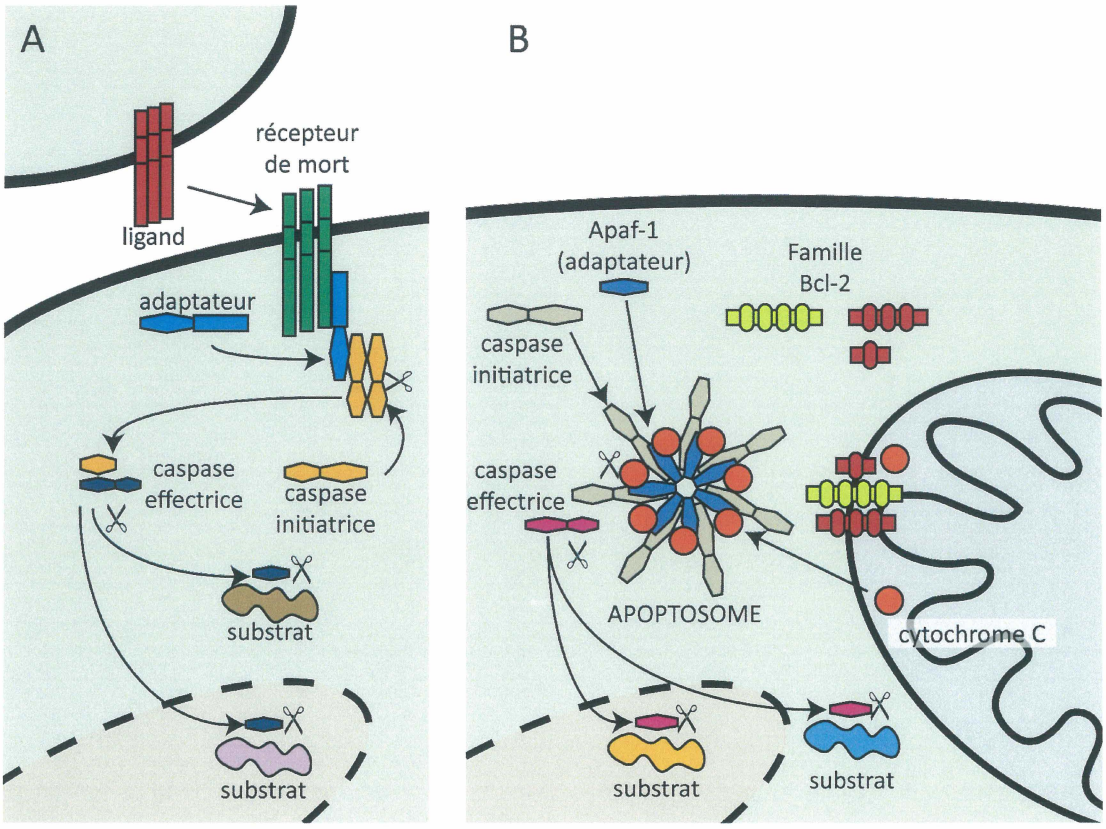

Figure 10 – Les voies de l'apoptose. *A*. La voie extrinsèque. Un ligand se lie à son récepteur, par exemple FasL à Fas. Ceci entraîne le clivage d'une pro-caspase initiatrice, l'activant. Celle-ci clivera et activera des caspases effectrices responsables du clivage de substrats cellulaires. *B*. La famille Bcl-2 régule la sortie de cytochrome C de la mitochondrie. Celui-ci peut former l'apoptosome avec Apaf-1 et une caspase initiatrice. L'apoptosome activera ensuite des caspases effectrices. Modifiée de Schwerk et Schulze-Osthoff, 2005.

Apaf-1 qui pourra ensuite se lier à la pro-caspase-9. Ceci constitue le point de non-retour de l'apoptose en formant le complexe nommé apoptosome qui activera ensuite plusieurs caspases effectrices (Degterev *et al.*, 2003). Il semble que les voies intrinsèques et extrinsèques ne sont pas exclusives puisque certaines des protéines impliquées sont présentes dans les deux. Par exemple, la caspase-8 activée par un récepteur de mort peut cliver et activer la protéine Bid qui sera relocalisée à mitochondrie et induira la sortie de cytochrome c (Luo *et al.*, 1998). La protéine anti-apoptotique Bcl-x_L peut prévenir cette sortie de cytochrome c (Gross *et al.*, 1999).

La régulation de l'apoptose due à ces différentes protéines est effectuée à plusieurs niveaux. Tel que mentionné, le clivage de certaines protéines jouera aussi un rôle majeur, majoritairement en jouant sur l'activation des caspases. De plus, la phosphorylation peut moduler l'activité de certaines protéines. Par exemple, Bcl-2 et Bcl-x_L peuvent être hyper-phosphorylées ce qui module ainsi leur activité (Bassik *et al.*, 2004; Tamura *et al.*, 2008) ou peut même entraîner la dégradation de Bcl-2 par le protéasome (Chadebech *et al.*, 1999). Un autre mécanisme majeur de régulation de l'apoptose est l'épissage alternatif.

L'apoptose et l'épissage alternatif

L'épissage alternatif joue un grand rôle à tous les niveaux de l'apoptose, formant des isoformes ayant des niveaux d'activités différents ou même antagonistes (Tableau 3, modifié de Schwerk et Schulze-Osthoff, 2005). Dans la voie extrinsèque, le ligand pro-apoptotique FasL possède un isoforme alternatif qui ne contient pas le domaine transmembranaire (Ayroldi *et al.*, 1999). Ce FasL soluble va inhiber l'apoptose. Le récepteur de mort Fas possède aussi plusieurs isoformes alternatifs qui antagonisent tous son activité pro-apoptotique (Cascino *et al.*, 1996; van Doorn *et al.*, 2002).

Outre le clivage, l'activité de la plupart des caspases peut aussi être contrôlée par l'épissage alternatif. Deux exemples bien étudiés sont la caspase-2 et la caspase-9. Dans le pré-mRNA de la caspase-2, l'exon 9 est alternatif. Son inclusion, favorisée par hnRNP A1, produit l'isoforme anti-apoptotique Casp2S (Jiang *et al.*, 1998). L'action des protéines SR ASF/SF2 et SC35 favorisera cependant son exclusion, formant l'isoforme pro-apoptotique Casp2L. Une séquence intronique (In100), liée par hnRNP I/PTB ainsi que le snRNP U2, inhibe l'épissage entre les exons 9 et 10, augmentant aussi la production de Casp2L (Cote *et al.*, 2001a; Cote

Protéine	Fonction cellulaire	Conséquences fonctionnelles de l'épissage alternatif
Ligands et récepteurs		
FasL	Activation de l'apoptose par voie des récepteurs de mort	Solubilité et potentiel apoptotique altérés
Fas	Apoptose, voie extrinsèque	Solubilité altéré, phénotype dominant négatif
LARD	Apoptose, voie extrinsèque (lymphocytes)	Solubilité et potentiel apoptotique altérés
Protéines adaptatrices et régulateurs		
TRAF2	Signalisation par récepteur TNF	Phénotype dominant négatif
TRAF3	Signalisation par récepteur TNF	Fonctions distinctes dans transduction des signaux
myDD88	Signalisation par récepteur Toll-like	Phénotype dominant négatif
MADD	Activation de MAPK	Fonctions antagonistes durant l'apoptose
Apaf-1	Composante de l'apoptosome	Fonctions antagonistes durant l'apoptose
Survivin	Régulation de l'apoptose et du cycle cellulaire	Localisation cellulaire et potentiel apoptotique différents
Smac/Diablo	Protéine mitochondriale liant IAP	Potentiel apoptotique altéré
Famille Bcl-2		
Bcl-x	Anti-apoptotique	Fonctions antagonistes durant l'apoptose
Bak	Pro-apoptotique	Potentiel apoptotique altéré (dépendant du type cellulaire)
Bid	Connexion entre la voie extrinsèque et intrinsèque	Potentiel apoptotique altéré
Bim	Pro-apoptotique (BH3 seulement)	Localisation cellulaire différente
Caspases et protéines similaires		
Caspase-2	Caspase initiatrice, clivage de substrats	Fonctions antagonistes durant l'apoptose
Caspase-9	Caspase initiatrice, clivage de substrats	Phénotype dominant négatif
Caspase-10	Caspase initiatrice, clivage de substrats	Activité différente
FLIP	Régulation de l'apoptose induite par les récepteurs de mort	Fonctions potentiellement antagonistes durant la réponse immunitaire
Substrats des caspases		
ICAD	Fragmentation de l'ADN apoptotique	Distribution cellulaire différente
CAD	Fragmentation de l'ADN apoptotique	
Acinus	Condensation de la chromatine apoptotique; épissage de l'ARN	
Famille p53		
p53, p63 et p73	Régulation de l'apoptose et du cycle cellulaire	Potentiel apoptotique altéré

Tableau 3 – L'implication de l'épissage alternatif dans l'apoptose. Quelques protéines impliquées dans l'apoptose qui possèdent des isoformes d'épissage alternatif. La fonction cellulaire de l'isoforme majoritaire est indiquée, ainsi que la conséquence de l'épissage alternatif. Modifié de Schwerk et Schulze-Osthoff, 2005.

et al., 2001b). L'épissage de la caspase-9 produit deux isoformes : la pro-apoptotique caspase-9a, contenant les quatre exons 3, 4, 5, et 6, ainsi que l'anti-apoptotique caspase-9b, qui exclut ces exons (Srinivasula *et al.*, 1999). L'ajout de l'agent chimiothérapeutique gemcitabine, un analogue de la cytidine causant l'arrêt de la réplication de l'ADN, augmente la concentration intracellulaire du lipide céramide, qui agirait sur ASF/SF2 et favoriserait la formation de l'isoforme pro-apoptotique (Chalfant *et al.*, 2002; Massiello et Chalfant, 2006). Plusieurs protéines adaptatrices possèdent des isoformes ayant des activités antagonistes. Apaf-1, une protéine centrale de l'apoptosome, peut être épissée alternativement en excluant un exon ce qui mènera à une activité anti-apoptotique (Benedict *et al.*, 2000). TRAF2 transmet l'activation du récepteur de la mort TNFR au facteur de transcription NF-κB, et une insertion d'un exon pour former TRAF2A donnera une protéine d'activité dominante négative.

Les gènes de la famille de protéines Bcl-2, contenant des membres anti-apoptotiques et pro-apoptotiques, sont très souvent épissés de façon alternative (Akgul *et al.*, 2004) menant souvent à une activité antagoniste. Bcl-2 possède deux isoformes alternatifs : Bcl-2α et Bcl-2β (Tsujimoto et Croce, 1986). La seule différence est l'excision d'une région dans l'ARNm de Bcl-2β, qui encode le domaine transmembranaire de la protéine. Ceci est suffisant pour mener à une protéine n'ayant pas l'activité anti-apoptotique caractéristique de Bcl-2α. Les deux isoformes connus de Mcl-1, soit Mcl-1L et Mcl-1S, ont une activité anti-apoptotique et pro-apoptotique respectivement (Bae *et al.*, 2000; Kozopas *et al.*, 1993). Bid a quatre isoformes, la moitié favorisant la survie de la cellule et l'autre la mort cellulaire (Renshaw *et al.*, 2004).

L'apoptose est donc très influencée par l'épissage alternatif, mais cette voie peut aussi affecter les facteurs d'épissage. En effet, plusieurs de ceux-ci sont des substrats des caspases (Fischer *et al.*, 2003). Les protéines hnRNPs A, C, I/PTB, K et R ainsi que KHSRP sont tous des substrats de clivage par diverses caspases, ce qui les inactive. Les kinases SRPK1 et SRPK2 sont aussi inactivés par le clivage, tandis que PP2A sera activée par clivage de son domaine de régulation. Finalement l'induction de l'apoptose par le récepteur Fas va entraîner le clivage de la portion protéique de 70 kDa du snRNP U1 (Casciola-Rosen *et al.*, 1994; Degen *et al.*, 2000).

Le cancer et l'épissage alternatif

La régulation aberrante de l'apoptose est une cause de la formation des cancers avec la prolifération, l'invasion et l'angiogenèse (Hanahan et Weinberg, 2000). L'épissage alternatif, jouant un rôle majeur dans la régulation des isoformes protéiques et de leurs activités, est donc un élément clé dans la formation des cancers (voir les revues de littérature suivantes : Carpenter *et al.*, 2006; Pajares *et al.*, 2007; Pettigrew et Brown, 2008; Revil *et al.*, 2006; Srebrow et Kornblihtt, 2006; Venables, 2006). Un épissage alternatif aberrant est aussi impliqué dans plusieurs maladies graves telles que la maladie de Parkinson, la fibrose cystique et la dystrophie myotonique (Buratti *et al.*, 2005; Kuyumcu-Martinez et Cooper, 2006; Singh *et al.*, 2004b; Singh, 2007; Tan *et al.*, 2005).

Plusieurs situations anormales peuvent mener à un épissage alternatif qui favorisera la formation de cancers. Les mutations de l'ADN peuvent causer l'inactivation ou la création de sites d'épissages ou de sites de liaison pour des facteurs d'épissage (Figure 11). Dans les

gènes BRCA1 et BRCA2, encodant pour deux suppresseurs de tumeurs, de nombreuses mutations des sites d'épissage ont été recensées qui augmentent les risques héréditaires de cancer du sein et/ou ovarien (Bonatti *et al.*, 2006; Chen *et al.*, 2006; Hoffman *et al.*, 1998; Liu *et al.*, 2001; Tesoriero *et al.*, 2005; Tommasi *et al.*, 2008). Aussi, au moins deux mutations dans ce gène peuvent détruire un ESE (Mazoyer *et al.*, 1998) entraînant potentiellement

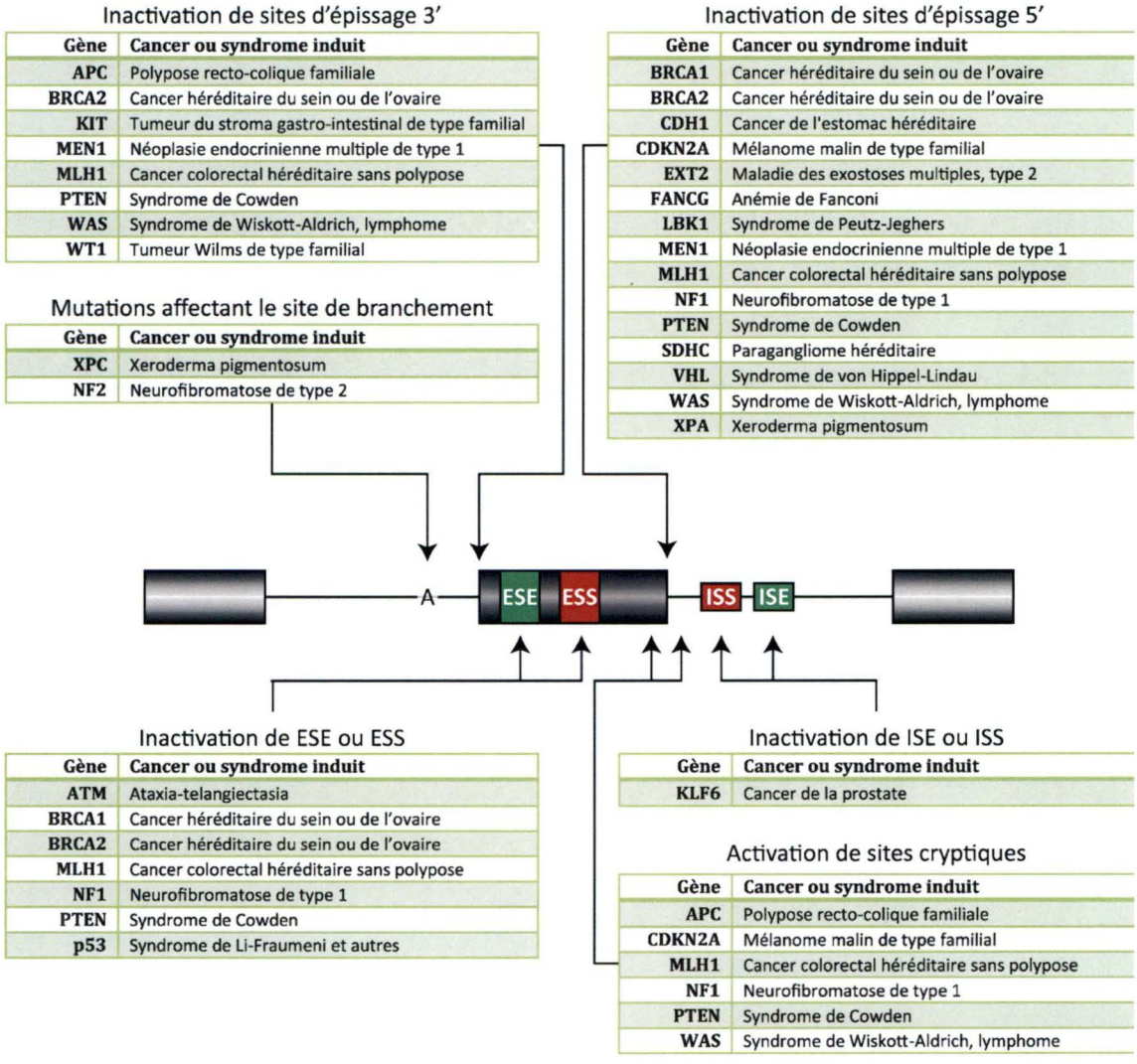

Figure 11 – Les défauts de l'épissage alternatif dans le cancer. Une liste des mutations d'ADN affectant différents aspects de l'épissage alternatif, ainsi que le cancer ou le syndrome induit. Modifié de Revil *et al.*, 2006; Shkreta *et al.* 2008.

l'exclusion d'un exon. Deux mutations dans la LI-cadhérine, identifiées dans des carcinomes hépatocellulaires, pourraient créer un ISS et un ESS causant l'exclusion de l'exon 7 et la création d'un codon d'arrêt de traduction prématuré.

Outre les mutations génomiques, la variation de l'activité des facteurs d'épissage peut aussi augmenter les risques de cancer en influençant l'épissage alternatif de pré-ARNm clés. Ceci peut s'effectuer par des changements dans la localisation cellulaire, la concentration ou les modifications post-traductionnelles des facteurs d'épissage. Durant la transition de cellules normales à adénocarcinomes mammaires, il y a augmentation de la concentration de protéines SR, dont SRp20, augmentant la formation d'isoformes de CD44 contenant des exons alternatifs (Stickeler *et al.*, 1999). Des niveaux élevés de certains hnRNPs, dont hnRNP A1, A2/B1, C1/C2, I/PTB et K, ainsi que des niveaux faibles de E1 et M4, sont retrouvés dans plusieurs types de cancers (voir la revue de littérature : Carpenter *et al.*, 2006). Il n'est cependant pas clair si et comment ces niveaux pourraient favoriser le développement des cancers cependant, tel qu'expliqué précédemment (voir « Les protéines hnRNPs », page 14), les protéines hnRNPs sont très impliquées dans l'épissage alternatif des gènes apoptotiques.

Dans certains cancers ovariens, il y a augmentation des niveaux des protéines SR SC35 et ASF/SF2 ainsi qu'une hausse de la phosphorylation de Tra2 et YB-1 (Fischer *et al.*, 2004). L'état de phosphorylation de Sam68 ainsi que hnRNP K sont importants aussi pour la croissance cellulaire anormale ainsi que le développement de tumeurs (Gorla *et al.*, 2006). La surexpression de la protéine kinase SRPK1, qui phosphoryle les protéines SR, est retrouvée dans certaines tumeurs et sa diminution de concentration par interférence à l'ARN sensibilise les cellules aux agents chimiothérapeutiques gemcitabine et cisplatin (Hayes *et*

al., 2006; Hayes *et al.*, 2007). Ainsi, la phosphorylation des facteurs d'épissage peut aussi jouer un rôle dans le développement de cancers, soit en modulant l'activité de ces protéines ou leur localisation cellulaire.

L'épissage alternatif peut même servir de marqueur de tissus cancéreux. La comparaison de l'épissage alternatif de 600 gènes associés au cancer, dans des tissus de cancer du sein ou normaux, a permis d'identifier 41 événements d'épissage alternatif qui étaient significativement différents entre ces deux tissus (Venables *et al.*, 2008a). La plupart de ces différences affectaient positivement la prolifération et la survie cellulaire, et ainsi le cancer. Dans un test à l'aveugle, il a été possible d'identifier les tissus cancéreux dans 96% des cas utilisant les douze meilleures cibles. Cette même analyse a été effectuée dans des tissus de cancer de l'ovaire, ce qui a identifié 48 événements d'épissage alternatif spécifiques à ceux-ci. En utilisant cette signature d'épissage alternatif de cancer, 80% des tissus ovariens cancéreux et tous les tissus normaux vérifiés pouvaient être identifiés dans une collection de 39 spécimens. (Klinck *et al.*, 2008). Le potentiel de l'épissage alternatif dans la détection de cancers est donc particulièrement intéressant.

L'épissage alternatif de bcl-x

L'épissage alternatif de bcl-x peut mener à deux isoformes majeurs et quelques isoformes mineurs (Figure 12), variant selon les types cellulaires et le développement.

Isoformes majeurs de *bcl-x*

L'isoforme le plus abondant dans la majorité des types cellulaires, surtout dans les cellules de cerveau, est Bcl-x_L (Boise *et al.*, 1993). Cet isoforme est formé lorsque le site d'épissage 5' proximal de l'exon 2 est utilisé (Figure 12). Ceci forme une protéine anti-apoptotique qui possède tous les quatre domaines d'homologie à Bcl-2 (BH). Le mécanisme d'action de celle-ci n'est pas encore complètement élucidé, mais il impliquerait une translocation aux membranes mitochondriale à laquelle Bcl-x_L sera accrochée grâce à son domaine transmembranaire (TM). Cette protéine peut lier les protéines pro-apoptotiques, dont Bak et Bax, l'inactivant ainsi. Cependant, lorsqu'elle est neutralisée par les protéines BH3, l'apoptose dépendante de Bak peut avoir lieu (van Delft et Huang, 2006). La surexpression de Bcl-x_L isoforme favorisant la survie de la cellule est retrouvée dans plusieurs types de tumeurs (Castilla *et al.*, 2006; Espana *et al.*, 2004; Linden *et al.*, 2004; Olopade *et al.*, 1997; Takehara *et al.*, 2001; Watanabe *et al.*, 2002; Xerri *et al.*, 1996). De plus, cette forte concentration de Bcl-x_L aide à protéger les cellules cancéreuses contre l'apoptose induite par différents traitements anti-cancer ou cytotoxiques (Castilla *et al.*, 2006; Lebedeva *et al.*, 2000; Li *et al.*, 2001; Oltersdorf *et al.*, 2005; Takehara *et al.*,2001; Wang *et al.*, 2006).

Lorsque le site d'épissage 5' distal est utilisé lors de l'épissage alternatif, il y a production de l'isoforme Bcl-x_S qui favorise la mort cellulaire (Figure 12) (Boise *et al.*, 1993; Clarke *et al.*, 1995; Ealovega *et al.*, 1996; Ohi *et al.*, 2000). La fonction moléculaire exacte de cette protéine n'est pas claire. Il se pourrait qu'elle agisse par formation d'hétérodimères avec Bcl-2 et Bcl-x_L (Minn *et al.*, 1996). Une molécule de Bcl-x_S est suffisante pour annuler l'effet de survie de quatre molécules de Bcl-x_L. La surexpression de Bcl-x_S sensibilise les cellules MCF-7,

exprimant fortement Bcl-x_L, à l'étoposide et au taxol, deux agents chimiothérapeutiques. La transfection d'oligomères de différents types qui bloquent le site d'épissage de x_L et force donc la formation de Bcl-x_S sensibilise aussi les cellules aux agents chimiothérapeutiques ou peut même induire l'apoptose (Mercatante *et al.*, 2001; Mercatante *et al.*, 2002; Taylor *et al.*, 1999). La liaison près du site d'épissage de Bcl-x_S d'un oligo chimérique d'acide nucléique peptidique (PNA) conjugué à huit répétitions de sérine-arginine (SR), augmente son utilisation et l'apoptose des cellules (Wilusz *et al.*, 2005). Dans la leucémie myéloïde aiguë, la perte de l'expression de Bcl-x_S dans

les cellules leucémiques est synonyme d'une diminution marquée de l'espérance de vie

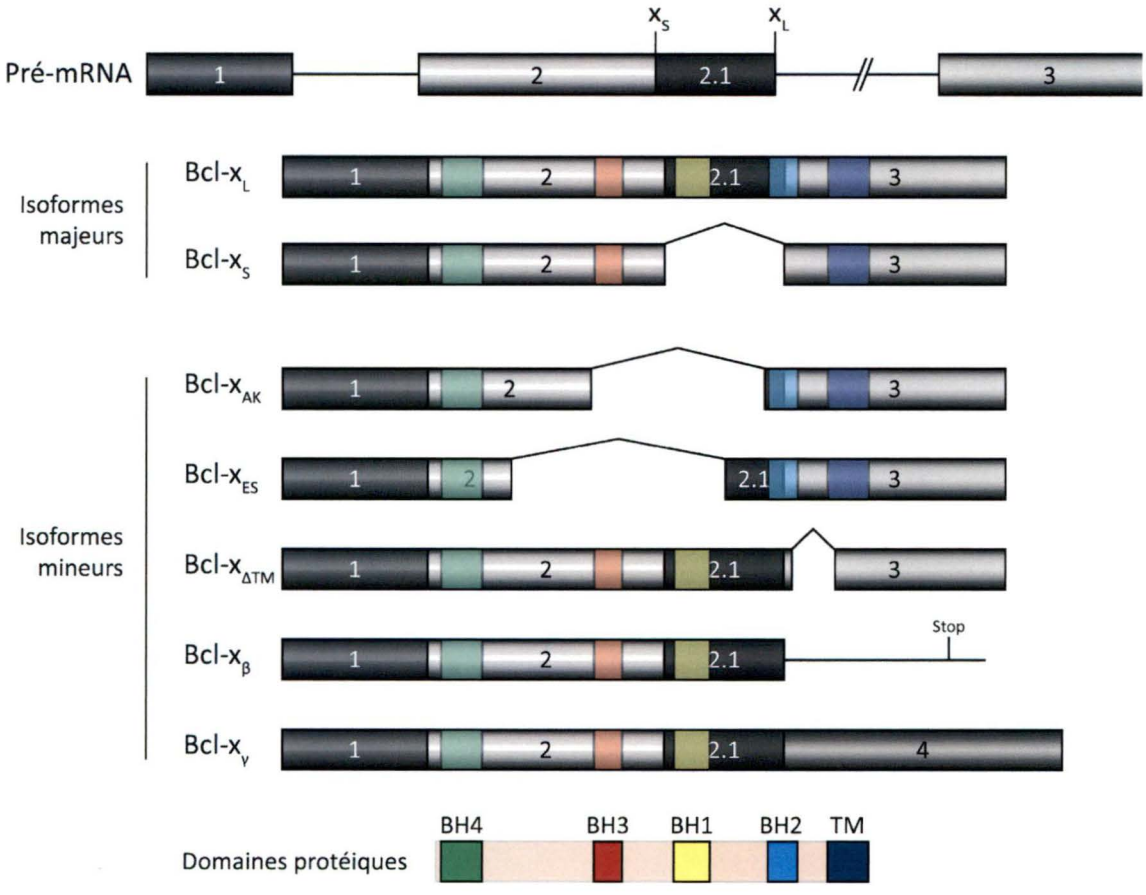

Figure 12 – Les isoformes d'épissage alternatif de *bcl-x*. L'épissage alternatif du pré-ARNm de bcl-x peut mener à sept isoformes connus, dont deux majoritaires. Les exons sont représentés par des boîtes et les introns par des lignes. Les domaines protéiques BH ainsi que le domaine TM sont en couleur pour démontrer l'effet de l'épissage alternatif sur la structure protéique.

(Yamaguchi *et al.*, 2002). Aucune mutation n'étant présente dans le gène de *bcl-x* de ces cellules, ce dérèglement de l'épissage serait probablement dû à des facteurs agissant en *trans*.

Isoformes mineurs de *bcl-x*

Bcl-x_{AK}, un isoforme mineur détecté dans la plupart des cellules, est formé lorsqu'il y a épissage à l'intérieur de l'exon 2 (Figure 12). Ceci entraîne la perte des domaines BH3 et BH1, cependant la surexpression de la protéine de 18 kDa induit l'apoptose dans les cellules de mélanomes quoique à un niveau moindre que Bcl-x_S (Hossini *et al.*, 2006).

Un autre isoforme encore plus court est Bcl-x_{ES} (13 kDa), produit par l'excision d'une portion de l'exon 2 et présent dans la plupart des types cellulaires (Schmitt *et al.*, 2004). Celui-ci a une activité anti-apoptotique médiée par son inhibition de la formation des oligomères Bax et d'Apaf-1. Bcl-x_{ES} contre donc le relâchement du cytochrome c, l'activation de la procaspase-9 et la cascade apoptotique qui s'ensuit.

Plusieurs nouveaux isoformes ont été trouvés chez les souris et rats. Puisque *bcl-x* est très conservé entre la souris, le rat et l'humain, il est probable que ceux-ci soit aussi exprimés chez l'humain. Par exemple, l'isoforme pro-apoptotique Bcl-x_β a d'abord été détecté chez le rat, dans le cœur, cervelet et thymus (Shiraiwa *et al.*, 1996), puis chez l'humain (Ban *et al.*, 1998) et la souris (Bianchini *et al.*, 2006). Cet isoforme est créé lors de l'absence d'utilisation des deux sites 5' de Bcl-x_S ou Bcl-x_L. L'intron encode donc une partie de la séquence

protéique. Dans les thymocytes murins, la dexaméthasone, un glucocorticoïde de synthèse, augmente le ratio Bcl-x_β/Bcl-x_L durant l'apoptose de ces cellules.

L'excision de 68 nt dans l'exon 3 par de nouveaux sites 5' et 3' internes forme Bcl-$x_{\Delta TM}$, présent dans la plupart des cellules de souris (Fang *et al.*, 1994). Le nouvel isoforme créé possède toutes les régions de Bcl-x_L mais une nouvelle séquence C-terminal au lieu du domaine transmembranaire. La protéine possède donc une activité anti-apoptotique mais n'est pas localisée à la mitochondrie et demeure dans le cytoplasme dans les cellules T et B immatures.

Dans les thymocytes et les cellules T matures de la souris, il y a expression de Bcl-x_γ qui protège de la mort induite par les récepteurs de cellules T (TCR) (Yang *et al.*, 1997). Cet isoforme est créé par l'utilisation du site d'épissage 5' de Bcl-x_L mais avec le site 3' d'un nouvel exon, menant donc encore seulement à une séquence C-terminale différente de Bcl-x_L.

Régulation de l'épissage alternatif de bcl-x

La régulation de l'épissage alternatif de ces différents isoformes a largement été étudié ces dernières années, avec une emphase particulière sur les deux isoformes les plus courants et mieux caractérisés : Bcl-x_L et Bcl-x_S. Plusieurs éléments ont été découverts ainsi que l'effet de différents promoteurs (Figure 13) ou des signaux cellulaires (Figure 14). Chez la souris (Figure 13A), cinq promoteurs ont été identifiés pour le gène de *bcl-x* (Grillot *et al.*, 1997; Pecci *et al.*, 2001). Les ratios des isoformes d'épissage alternatif varient selon le promoteur initiant la

transcription, et différents signaux peuvent potentiellement influencer le choix du promoteur. Par exemple, les stéroïdes peuvent augmenter l'utilisation du promoteur P4 qui possède deux éléments de réponse aux hormones (HRE), et ceci augmenterait la production de l'isoforme Bcl-x_L (Viegas *et al.*, 2004). Ceci est similaire à l'effet induit par le glucocorticoïdes dexaméthasone dans les cellules de fibrosarcome (Gascoyne *et al.*, 2003). Cependant, ce même glucocorticoïde a l'effet contraire chez les cellules lymphoïdes, réprimant l'utilisation du promoteur P4, augmentant la production de l'isoforme Bcl-x_S et favorisant l'apoptose (Rocha-Viegas *et al.*, 2006).

Chez l'humain, seulement trois promoteurs ont été découverts jusqu'à présent (Figure 13B) (MacCarthy-Morrogh *et al.*, 2000). Ets, un facteur de transcription, et le ligand pour CD40 (CD40L), activent chacun un promoteur différent dans les lymphocytes malins (Habens *et al.*,

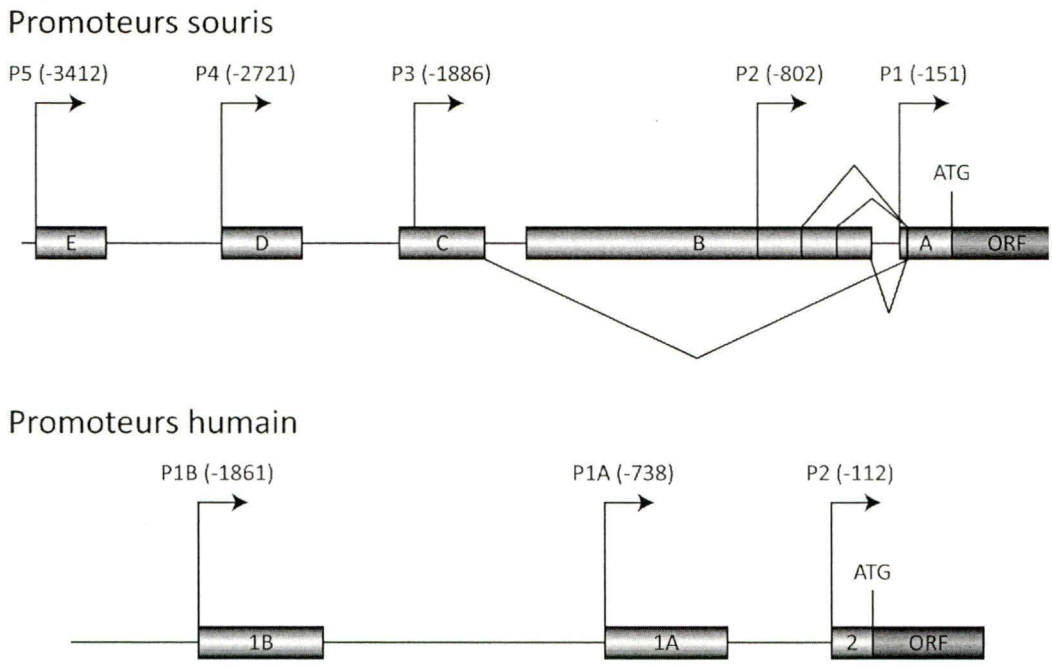

Figure 13 – Les promoteurs de *bcl-x*. Le gène de bcl-x possède cinq promoteurs connus chez la souris et trois chez l'humain. La transcription par ces différents promoteurs entraîne un ratio d'épissage caractéristique à chacun. Modifié de Viegas *et al.*, 2004; Habens *et al.*, 2007.

2007). Il n'est pas clair si et comment ces promoteurs régulent l'épissage de bcl-x. Ils pourraient agir sur la vitesse de transcription de la polymérase II ou au recrutement différentiel des facteurs d'épissage, que ce soit directement au site de transcription ou par une phosphorylation différente de la queue C-terminale de la polymérase.

Sur le pré-ARNm de *bcl-x* (Figure 14), des séquences présentes dans l'intron (IRE) sont responsables de l'effet induit par l'interleukin-6 (IL-6) et le « granulocyte-macrophage

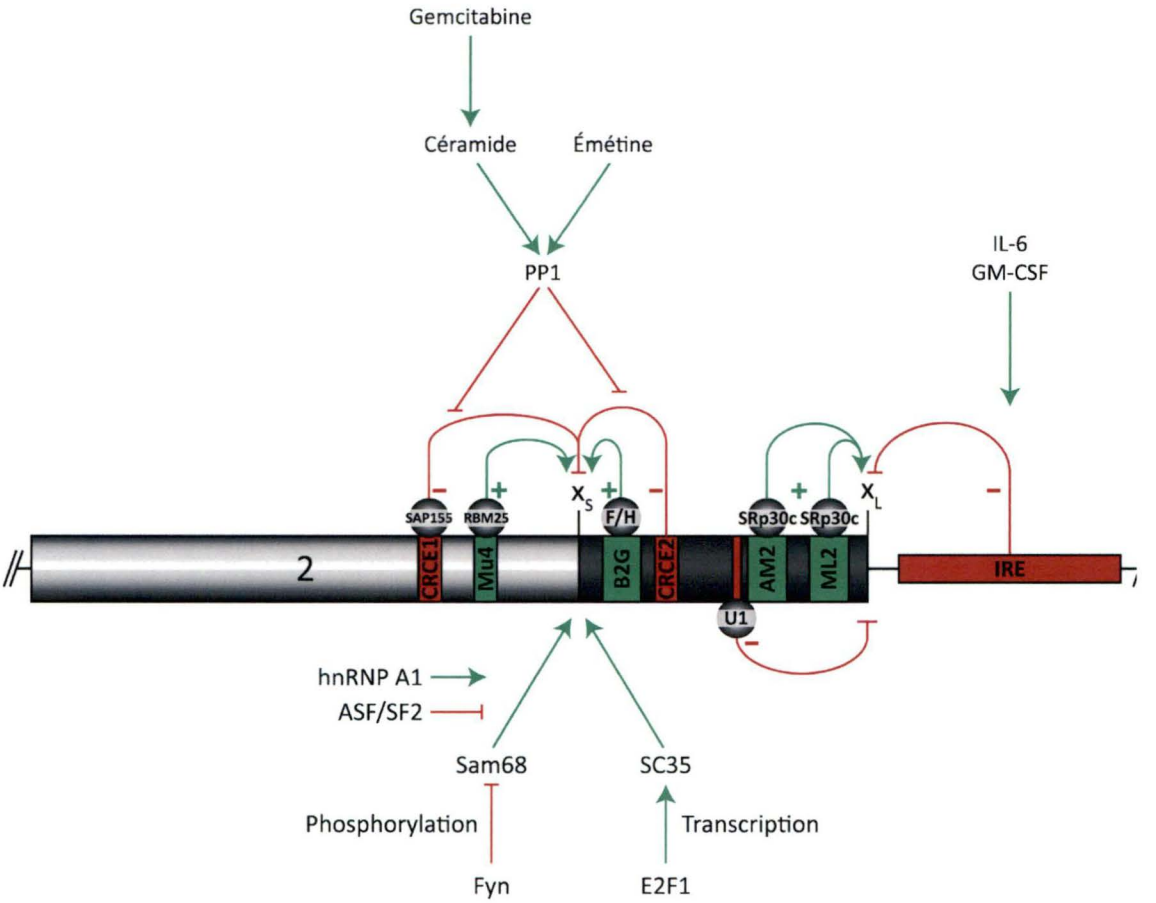

Figure 14 – La régulation de l'épissage alternatif de *bcl-x*. La sélection des deux sites d'épissage 5' est régulée par de nombreux éléments, facteurs et voies. IRE dans l'intron médie l'effet des cytokines IL-6 et GM-CSF. Les éléments AM2 et ML2 sont liés par SRp30c qui augmente l'utilisation de x_L, tandis que U1 lie deux sites cryptiques et le diminue. CRCE1 et CRCE2, le premier lié par SAP155, inhibe le site d'épissage x_S, médié par la protéine phosphatase 1 (PP1) sous l'effet de la céramide. L'émétine influence aussi la PP1, mais il n'est pas clair par quel facteur celle-ci agit. RBM25, lié en amont de x_S, et les protéines hnRNPs F/H, liées à B2G en aval, augmentent la production de l'isoforme Bcl-x_S. La phosphorylation de Sam68 par Fyn augmente aussi cette isoforme, ceci est dépendant de hnRNP A1 tandis qu'ASF/SF2 l'inhibe. L'augmentation de la transcription de la protéine SR SC35 par le facteur de transcription E2F1 stimule l'utilisation de x_S.

colony stimulating factor » (GM-CSF). Ces deux cytokines augmentent la production de l'isoforme Bcl-x_S dans une lignée de cellules de leucémie. Dans la lignée cellulaire d'adénocarcinome de poumon, A549, l'agent chimiothérapeutique gemcitabine accroît aussi l'isoforme Bcl-x_S en triplant la concentration cellulaire de céramide (Chalfant *et al.*, 2002). Ce lipide induit l'apoptose en réponse à plusieurs autres agents anti-cancer et module aussi l'épissage de la caspase-9. Il agirait en activant la protéine phosphatase-1 (PP1) qui peut déphosphoryler les protéines SR. Deux régions sur le pré-mRNA de bcl-x ont été identifiées comme nécessaires pour l'effet induit sur l'épissage par la céramide, soit les « ceramide-responsive RNA *cis*-element » 1 et 2 (CRCE-1, riche en purines, et CRCE-2, riche en pyrimidines) (Massiello *et al.*, 2004). Celles-ci sont situées en amont et en aval du site 5' de Bcl-x_S, respectivement, et inhiberaient l'utilisation de ce site. CRCE-1 serait liée par la protéine SAP155, un facteur d'épissage faisant partie du complexe essentiel SF3b qui s'associe au snRNP U2 lors de l'épissage constitutif (Massiello *et al.*, 2006). La céramide, un lipide bioactif naturellement présent dans les membranes cellulaires, pourrait moduler l'état de phosphorylation de ce facteur et ainsi réguler son activité sur le site d'épissage x_S. Les phosphatases PP1 et PP2A ont déjà été démontrées comme déphosphorylant SAP155 durant la transition entre la première étape et la deuxième étape de l'épissage (Shi *et al.*, 2006).

Un autre facteur d'épissage influençant l'épissage de bcl-x modulé par la phosphorylation est Sam68 (« Src-associated in mitosis 68 kDa ») (Paronetto *et al.*, 2007). Cette protéine fait partie de la famille STAR (« Signal Transduction and Activation of RNA metabolism »), dont les membres sont impliqués dans la transduction des signaux pour la maturation des ARN (Lukong et Richard, 2003). La concentration cellulaire de Sam68 influence l'apoptose et le cycle cellulaire (Taylor *et al.*, 2004). La phosphorylation de cette protéine par les kinases

Erk1/2 augmente l'inclusion de l'exon v5 dans l'ARNm de CD44, un récepteur impliqué dans l'adhésion et la migration cellulaire (Matter *et al.*, 2002). Cependant, c'est plutôt la phosphorylation de Sam68 par la kinase Fyn qui influence l'épissage alternatif de bcl-x. Ceci inhibe la liaison de Sam68 à un site, pour l'instant non-défini, ainsi que son augmentation de l'isoforme Bcl-x_S. L'effet positif de Sam68 sur cet isoforme est dépendant d'une coopération possible avec hnRNP A1, mais est contré par ASF/SF2. L'action de ASF/SF2 pourrait entre autres expliquer les observations précédentes qui l'impliquaient dans la protection contre l'apoptose (Li *et al.*, 2005). Étonnement, dans les lignées cellulaires de cancer, Sam68 est acétylé ce qui augmenterait sa liaison à l'ARN (Babic *et al.*, 2004). Cette incongruité pourrait être due à une affinité différente de cette protéine selon son état de phosphorylation et d'acétylation.

La transcription est aussi un mécanisme de régulation indirect de l'épissage alternatif de bcl-x. E2F1 est un facteur de transcription qui a un rôle clé durant l'apoptose et le cycle cellulaire. Des agents endommageant l'ADN induisent une augmentation de la transcription du gène de la protéine SR SC35 par E2F1. La hausse de la concentration de SC35 va induire une augmentation des isoformes pro-apoptotiques de plusieurs gènes apoptotiques clés tels que c-flip, caspase-8 et -9 ainsi que bcl-x par un mécanisme encore inconnu (Merdzhanova *et al.*, 2008).

Récemment, il a été démontré que la protéine RBM25 augmente l'utilisation du site d'épissage Bcl-x_S par la liaison à un élément CGGGCA (Mut4) situé en amont de ce site. Ceci corrèle avec le fait que la surexpression de cette protéine induit l'apoptose. RBM25 pourrait

agir en stabilisant le snRNP U1 au site d'épissage 5' de Bcl-x_S de par son interaction avec hLuc7a, une protéine associé à ce snRNP (Zhou *et al.*, 2008).

Finalement, depuis quelques années j'ai contribué à l'identification de plusieurs éléments et facteurs influençant l'épissage alternatif de bcl-x. Premièrement, les protéines hnRNP F et H peuvent lier la région B2 et ainsi favoriser l'utilisation du site Bcl-x_S situé en amont (Garneau *et al.*, 2005, voir Annexe 1). Cette région, d'une longueur de 77 nt, contiendrait plusieurs triplets ou quadruplets de guanidines, des sites de liaison connus pour hnRNP F et H. La majorité est contenue dans le sous-élément B2G, de 30 nt, d'ailleurs responsable de l'activité de B2. Étonnement, hnRNP F et H sont souvent surexprimés dans les tumeurs (Balasubramani *et al.*, 2006; Rauch *et al.*, 2004) alors que l'abondance nucléaire de hnRNP H est aussi plus élevé dans ces tissus que normal (Honore *et al.*, 2004). Ceci est l'opposé de ce que l'on s'attendrait de protéines augmentant l'isoforme pro-apoptotique Bcl-x_S, mais il se peut que l'activité de ces protéines ne soit pas modulée que par leur concentration mais aussi par leur état de phosphorylation. En effet, durant l'apoptose des cellules HL-60, il y a phosphorylation de hnRNP H (Navakauskiene *et al.*, 2004).

Dans la région B3, les protéines SR SRp30c peuvent lier les éléments AM2 et ML2, situé en amont de site d'épissage de Bcl-x_L et favoriser l'utilisation de ce dernier (Cloutier *et al.*, 2008, voir Annexe 2). La protéine SRp30c est surexprimée dans les cellules T activées (Screaton *et al.*, 1995). Ainsi, la présence de cette protéine favorisant l'isoforme anti-apoptotique pourrait permettre à ces cellules de survivre une fois la sélection négative effectuée dans le thymus (Strasser et Pellegrini, 2004). De plus, il y a dans la région B3 une séquence ayant l'activité contraire des deux éléments précédents. Celle-ci contiendrait deux sites d'épissage

5' cryptiques pouvant être liés par le snRNP U1. Ceci inhiberait l'utilisation du site d'épissage 5' classique de Bcl-x_L d'une manière non encore élucidée, possiblement par la formation de complexes d'épissage non-fonctionnels sur les sites cryptiques.

Le but de mon doctorat était d'augmenter la compréhension de l'épissage alternatif du gène apoptotique *bcl-x*. Outre ma participation aux deux études précédentes sur les régions B2 et B3, j'ai aussi entrepris d'élucider le mécanisme d'action d'une autre région nouvellement identifiée : B1. Finalement, j'ai étudié le mécanisme d'action de la staurosporine sur l'épissage de *bcl-x*. Cet alcaloïde, initialement isolé des bactéries, inhibe une large gamme de protéine kinases avec beaucoup d'affinité en empêchant la liaison de l'ATP à celles-ci. J'ai identifié la kinase inhibée influençant l'épissage de bcl-x comme étant la protéine kinase C (PKC), qui agit possiblement en phosphorylant un facteur d'épissage. J'ai aussi identifié la région par laquelle agit ce facteur potentiel, soit la région SB1.

Chapitre I

Revil, T., Toutant, J., Shkreta, L., Garneau, D., Cloutier, P., et Chabot, B. 2007. Protein kinase C-dependent control of Bcl-x alternative splicing. *Molecular and Cellular Biology* **27**(24): 8431-8441.

Préambule

L'épissage alternatif de *bcl-x* génère la protéine pro-apoptotique Bcl-x_S et l'isoforme anti-apoptotique Bcl-x_L. Cet épissage est couplé à des signaux de transduction puisque la céramide, les hormones et facteurs de croissance modulent le ratio des isoformes de *bcl-x* dans différentes lignées cellulaires. Nous démontrons ici que l'inhibiteur de la protéine kinase C (PKC) et inducteur d'apoptose, staurosporine favorise la formation de l'isoforme d'ARNm x_S dans les cellules 293. L'augmentation de Bcl-x_S induite par la staurosporine implique probablement des évènements de signalisation qui affectent l'épissage puisqu'elle nécessite une transcription active, aucune synthèse protéique et est indépendante de l'activation des caspases. De plus, l'augmentation de Bcl-x_S est reproduite avec des inhibiteurs plus spécifiques de PKC. L'épissage alternatife du récepteur tyrosine kinase *Axl* est affecté de manière similaire par la staurosporine dans les cellules 293. Contrairement à cette lignée cellulaire, les inhibiteurs de PKC n'influencent pas l'épissage de *bcl-x* ni d'*Axl* dans des lignées cellulaires de cancer, suggérant que ces lignées cellulaires ont subi des altérations déconnectant les décisions d'épissage de la signalisation PKC-dépendante. Utilisant des mini-gènes, nous démontrons qu'une région exonique située en amont du site

d'épissage 5' de Bcl-x_S est importante afin de moduler l'effet de la staurosporine sur l'épissage de *bcl-x*. Quand des parties de cet élément sont placées dans d'autres unités d'épissage, elles reproduisent l'effet de modulation dépendant de l'ajout de la staurosporine, suggérant la présence de facteurs qui couplent les décisions d'épissage et la signalisation PKC.

J'ai réalisé toutes les expériences dans cet article sauf celles de la figure 3A (Johanne Toutant) et 5B (Lulzim Shkreta et Philippe Cloutier). Daniel Garneau a été le premier à observer l'effet de la staurosporine sur l'épissage alternatif de *bcl-x*. J'ai aussi contribué à l'écriture de cet article.

Protein Kinase C-dependent Control of *Bcl-x* Alternative Splicing

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Running title: Control of *Bcl-x* splicing by PKC

ABSTRACT

The alternative splicing of *Bcl-x* generates the pro-apoptotic Bcl-x_S protein and the anti-apoptotic isoform Bcl-x_L. *Bcl-x* splicing is coupled to signal transduction since ceramide, hormones and growth factors alter the ratio of the *Bcl-x* isoforms in different cell lines. Here we report that the protein kinase C (PKC) inhibitor and apoptotic inducer staurosporine switches the production of *Bcl-x* towards the x_S mRNA isoform in 293 cells. The increase in Bcl-x_S elicited by staurosporine likely involves signaling events that affect splicing decisions because it requires active transcription, no new protein synthesis, and is independent of

caspases activation. Moreover, the increase in Bcl-x_S is reproduced with more specific inhibitors of PKC. Alternative splicing of the receptor tyrosine kinase *Axl* is similarly affected by staurosporine in 293 cells. In contrast to 293 cells, PKC inhibitors do not influence the alternative splicing of *Bcl-x* and *Axl* in cancer cell lines, suggesting that these cells have sustained alterations that uncouple splicing decisions from PKC-dependent signaling. Using minigenes, we show that an exonic region located upstream of the Bcl-x_S 5' splice site is important to mediate the staurosporine shift in *Bcl-x* splicing. When transplanted to other alternative splicing units, portions of this region confer splicing modulation and responsiveness to staurosporine, suggesting the existence of factors that couple splicing decisions with PKC signaling.

INTRODUCTION

Alternative splicing of pre-messenger RNAs (pre-mRNAs) provides a powerful mechanism to augment the protein repertoire encoded by metazoan genomes. It is estimated that 74% of all multi-exonic human genes are alternatively spliced (Johnson *et al.*, 2003). Moreover, alternative splicing is becoming increasingly relevant to a variety of human diseases, including cancer (Novoyatleva *et al.*, 2006; Srebrow et Kornblihtt, 2006). These observations justify current efforts devoted at uncovering the basic principles of alternative splicing control. Many studies have provided valuable insights into the role of specific elements and factors in splicing modulation (Black, 2003; Matlin *et al.*, 2005; Smith et Valcarcel, 2000). Proteins that bind to specific sequence elements to affect splice site selection include SR proteins, hnRNP proteins and other related RNA binding proteins like TIA-1, ETR-3, Raver-1 and Sam68 (Charlet *et al.*, 2002; Gromak *et al.*, 2003; Izquierdo *et al.*, 2005; Martinez-

Contreras *et al.*, 2007; Matter *et al.*, 2002; Sanford *et al.*, 2003). Many of these proteins can be modified post-translationally, and some of the modifications, like phosphorylation, can affect their localization and activity (Matter *et al.*, 2002; Shin et Manley, 2004).

Although we expect that the coupling between signal transduction events and alternative splicing decisions will represent a major network of regulation, very little is known about how splicing is coordinated by a variety of effectors to attune cells to specific environmental demands and stresses. Possibly the first system describing a link between signal transduction and splicing involved the cell surface molecule CD44 in which exon v5 inclusion was stimulated following activation of the protein kinase C (PKC) or ERK, as part of the Ras signaling pathway (Konig *et al.*, 1998). It was later shown that this occurred through stimulation of Sam68 binding to exon v5 after its phosphorylation (Matter *et al.*, 2002). Interestingly, the Ras-induced production of CD44 receptors made from the exon v6-containing mRNA in turns enhances Ras activation to enforce cell cycle progression (Cheng *et al.*, 2006).

A signaling pathway also triggered by Ras and PKC promotes the skipping of exons in the transmembrane protein phosphatase CD45 pre-mRNA (Lynch et Weiss, 2000; Rothrock *et al.*, 2003). Because hnRNP L is essential for the repression of several CD45 regulated exons (Rothrock *et al.*, 2005), the aim of this signaling route may be to enforce the activity of hnRNP L or to repress the activity of proteins bound to nearby enhancers (Lemaire *et al.*, 1999; ten Dam *et al.*, 2000). Recent progress has also been made in identifying sequences and factors that control alternative splicing decisions of the NMDA receptor upon depolarization or constitutive CamK IV expression (Ares, 2007). The instruction to skip exon

C1 is relayed by a variety of elements, some bound by hnRNP A1 (An et Grabowski, 2007; Lee *et al.*, 2007). On the other hand, the cellular localization of hnRNP A1 is controlled by the $MEK_{3/6}$-p38 pathway (van der Houven van Oordt *et al.*, 2000). SR proteins have been implicated in multiple cases of alternative splicing control and they are extensively phosphorylated. Although SR proteins can be phosphorylated by SRPK1, SRPK2, Clk/Sty and topoisomerase I (Graveley, 2000), the signaling pathways that converge on these kinases remain unclear. Recently, contributions to splicing control by signaling pathways involving AKT kinase, phosphatidylinositol 3-kinase and JNK have also been documented (Blaustein *et al.*, 2004; Blaustein *et al.*, 2005; Ghosh *et al.*, 2007; Patel *et al.*, 2005; Patel *et al.*, 2006; Pelisch *et al.*, 2005).

Apoptosis or programmed cell death represents an important mechanism that equips cells with the ability to respond dramatically to external and internal insults. Consequently, apoptosis must be tightly connected to a variety of signals that are transducing information on the status of the cell and its environment (Schwerk et Schulze-Osthoff, 2005). In human cells, apoptosis implicates a large number of factors including transcriptional regulators, transmembrane receptors, cytoplasmic mediators, adaptors, and caspases. Alternative splicing plays a major role in the control of apoptosis (Schwerk et Schulze-Osthoff, 2005; Shin et Manley, 2004; Wu *et al.*, 2003). In many cases, alternative splicing programs the synthesis of proteins with different and sometimes opposing function in apoptosis. This contribution affects all categories of apoptotic factors (Schwerk et Schulze-Osthoff, 2005). For example, the production of soluble forms of Fas and FasL can have an anti-apoptotic function (Ayroldi *et al.*, 1999; Cascino *et al.*, 1996). Many members of the Bcl-2 family of proteins are also subject to splicing regulation, with *Bcl-x* possibly representing the best-documented case.

The Bcl-x_L splice isoform is produced through the use of a specific 5' splice site in exon 2 (Fig. 1A). One manner by which Bcl-x_L exerts its anti-apoptotic activity is by binding to and inhibiting pro-apoptotic BH3 proteins such as Bad or Bax (Cheng *et al.*, 2001). In contrast, Bcl-x_S displays pro-apoptotic activity and is derived from the use of a 5' splice site located 189 nt upstream from the x_L site. Bcl-x_S forms active homodimers and heterodimers with pro-apoptotic Bcl-2 family members (Lindenboim *et al.*, 2001). Alternatively, Bcl-x_S can heterodimerize with anti-apoptotic Bcl-2 family members such as Bcl-x_L, rendering them inactive (Chang *et al.*, 1999; Lindenboim *et al.*, 2001).

Despite the anticipated coupling between signal transduction events and alternative splicing decisions in apoptotic genes, there are only a few cases of this type of cross-talk that have been reported. Activation of Fas at the membrane changes the alternative splicing of caspase-9 and *Bcl-x* (Chalfant *et al.*, 2001; Chalfant *et al.*, 2002). It turns out that Fas activation and a variety of other stimuli (Ballou *et al.*, 1992; Cifone *et al.*, 1994; Hannun, 1994; Kim *et al.*, 1991; Liscovitch, 1992; Okazaki *et al.*, 1989; Quintans *et al.*, 1994) promote the production of ceramide, a regulator of stress responses and growth pathways. Ceramide activates protein phosphatase PP1, which can dephosphorylate SR proteins (Chalfant *et al.*, 2001). Two cis-acting elements (CRCE1 and CRCE2) are necessary for the ceramide-induced accumulation of Bcl-x_S in A549 cells (Fig. 1B) (Massiello *et al.*, 2004). CRCE1 is bound by the U2-associated spliceosomal complex protein SAP155 (Massiello *et al.*, 2006), which is a substrate for PP1/PP2A phosphatases (Shi *et al.*, 2006). S-adenosylmethionine and its metabolite 5' methylthioadenosine also increase PP1 expression and Bcl-x_S production in HepG2 and 293 cells (Yang *et al.*, 2004). A role for SR protein in *Bcl-x* splicing regulation may also be predicted based on the effect of an inhibitor of the SR protein kinase topoisomerase I

(Pilch *et al.*, 2001). The ratio of the *Bcl-x* isoforms is also affected by growth factors including IL1alpha (Pollock *et al.*, 2003), IL-6 and GM-CSF (Li *et al.*, 2004). TPA can also affect the alternative splicing of *Bcl-x* in an apparent PKC-dependent manner (Li *et al.*, 2004). A large intronic region immediately downstream from the Bcl-x_L 5′ splice site (Fig. 1B) has been linked to the effects of TPA and cytokines (Li *et al.*, 2004). Very recently, phosphorylation of Sam68 by Fyn was shown to stimulate Bcl-x_S usage in HEK293 cells (Paronetto *et al.*, 2007). The same study reported that hnRNP A1 can cooperate with Sam68 but that ASF/SF2 antagonizes the activity of Sam68 on *Bcl-x* splicing.

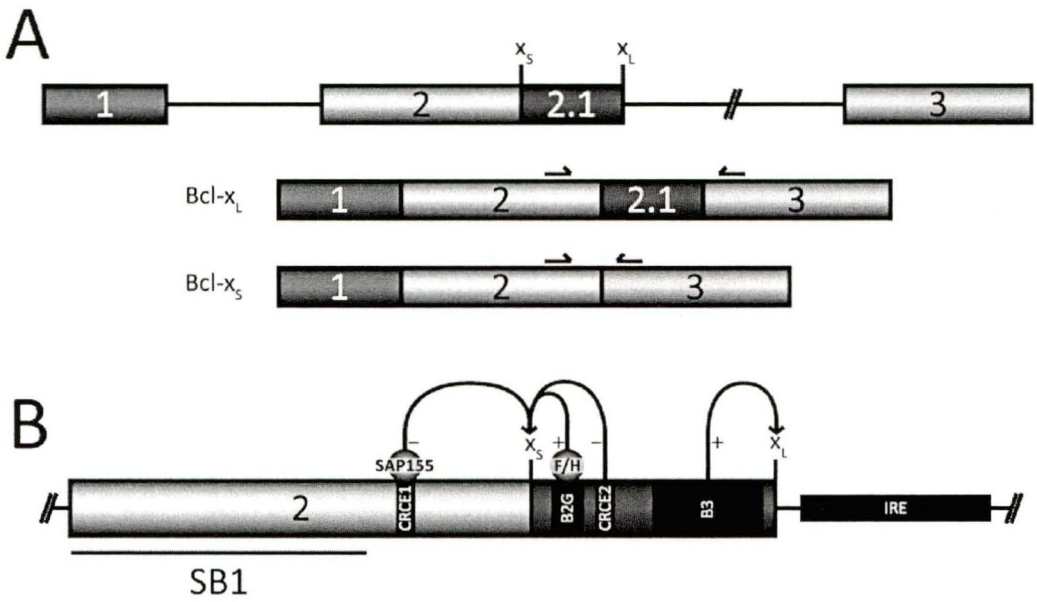

FIG. 1. Alternative splicing of the *Bcl-x* pre-mRNA. (A) The *Bcl-x* pre-mRNA is alternatively spliced to produce two major isoforms, Bcl-x_L and Bcl-x_S. The position of the primers used in RT-PCR assays is shown. (B) Sequences and factors documented to affect the alternative splicing of *Bcl-x*. The CRCE1 and CRCE2 elements modulate the response of A549 cells to ceramide that activates the use of the 5′ splice site of Bcl-x_S. (Massiello *et al.*, 2004). CRCE1 is bound by the U2 snRNP protein SAP155, and its knockdown increases the production of Bcl-x_S (Massiello *et al.*, 2006). B2G is bound by hnRNP H and stimulates the production of Bcl-x_S (Garneau *et al.*, 2005). B3 enhances the use of the Bcl-x_L 5′ splice site (Garneau *et al.*, 2005). The intron regulatory element (IRE) has been implicated in mediating the activity of growth factors and TPA on *Bcl-x* splicing in various cell lines (Li *et al.*, 2004). The SB1 element was previously shown to decrease the production of the Bcl-x_S isoform (Garneau *et al.*, 2005) and its position is indicated. Sam68 and hnRNP A1 also contribute to *Bcl-x* splicing but the *cis*-acting elements mediating these effects have not yet been mapped (Paronetto *et al.*, 2007).

Also interesting is the observation that glucocorticoids activate transcription of *Bcl-x* from only one of five mouse promoters (Viegas *et al.*, 2004). Mouse promoter P4 favors the production of the Bcl-x_L splice isoforms (Pecci *et al.*, 2001), whereas human promoter 1 displays this characteristic in human hepatoma cells (Yang *et al.*, 2004). Thus, transcriptional events can also determine the balance of pro- and anti-apoptotic *Bcl-x* isoforms.

Given the crucial role of *Bcl-x* in apoptosis and the ability of its pre-mRNA to respond to various classes of effectors, we have devoted efforts towards understanding regulatory aspects of *Bcl-x* splicing control. Our previous work uncovered an element bound by the hnRNP H protein that enforces the use of the upstream Bcl-x_S 5' splice site (Garneau *et al.*, 2005) (Fig. 1B). In addition, an enhancer element was located immediately upstream of the Bcl-x_L 5' splice site (Garneau *et al.*, 2005). Here, we report that staurosporine improves the production of the Bcl-x_S isoform by interfering with a signaling pathway dependent on PKC. The impact of staurosporine on *Bcl-x* splicing is mediated through a region in exon 2 (SB1) that normally represses the production of Bcl-x_S. While staurosporine lifts this repression in 293 cells, it is unable to do so in a variety of cancer cell lines. Our results therefore uncover a novel regulatory mechanism affecting the alternative splicing of Bcl-x. Alterations in this pathway may contribute to the greater resistance of cancer cells to apoptosis-inducing agents.

MATERIALS AND METHODS

Cell culture. The 293 cells used in this study were the EcR-293 cell line (Invitrogen). EcR-293, HeLa, U373 and U-87 cells were maintained in Dulbeccos' modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% glutamine. PC-3 cells were maintained in Ham's-F12 medium containing 10% fetal bovine serum and 1% glutamine. SKOV-3 cells were maintained in DMEM/F12 medium containing 10% fetal bovine serum and 1% glutamine. MCF7 cells were maintained in EMEM/Earle's BSS medium containing 10% fetal bovine serum, 1% glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 0.01 mg/ml bovine insulin. HCT 116 cells were maintained in McCoy's 5a medium containing 10% fetal bovine serum and 1% glutamine.

Drug treatment and RT-PCR analysis. 3 x 10^5 cells of EcR-293, 2 x 10^5 cells of HeLa or MCF-7, 1.3 x 10^5 cells of U-87, 1.5 x 10^5 cells of SKOV-3, HCT 116 or U373 were plated in 35 mm^2 wells. Forty-eight hours later, 25 or 50 nM of staurosporine (Roche or Calbiochem) or the indicated concentrations of Gö6976 (Calbiochem) or calphostin C (Calbiochem) in the presence of light, was added for the indicated times. One hundred µM of z-VAD-fmk (Calbiochem) or the indicated concentrations of 5, 6-Dichloro-1-b-D-ribofuranosylbenzimidazole (DRB) (Calbiochem) or cycloheximide (Calbiochem) were added 1 h before addition of staurosporine. Analysis of splicing profiles of *Bcl-x* was done as described previously (Garneau *et al.*, 2005). *Axl* splicing products were amplified with primers Axl-f (CCCCTGAGAACATTAGTGCT) and Axl-r (AGAGCCAAGATGAGGACACA) using the same procedure and conditions than for *Bcl-x*.

Plasmid constructs. The *Bcl-x* minigenes were constructed as described previously (Garneau *et al.*, 2005). Portions of SB1 were amplified by PCR using the following primers: for A, A-forw (ATGCTGCAGTCGAGCTTCAG) and A-rev (CATCTGCAGAACCACCAGC); for B, B-forw (ATGCTGCAGAACCAGAGAC) and B-rev (CATCTGCAGCTCAGTCCTG); and for C, C-forw (ATGCTGCAGACTTTCTCTC) and C-rev (TATCTGCAGGGGCTGTCTG). Each PCR product was digested by PstI and purified on gel. They were then ligated into Dup51 previously digested with NsiI. The plasmid constructs were verified by enzymatic digestion and sequencing. pCMV-E1a plasmid has been described previously. The E1a minigene was constructed by digesting pCMVE1a (Yang *et al.*, 1994) with PstI, blunting and then digesting by XbaI. The E1a fragment was gel-purified and inserted in pcDNA3.1+ digested with EcoRV and XbaI. Fragments B and C of SB1 were then inserted into the blunted EcoRI site.

Transfection of 293 cells. 2 x 10^5 cells were plated in 35 mm^2 wells. Forty-eight hours later, 0.05 µg of DNA and 4 µl of PEI (1 µg/µl) were incubated 15 min in 200 µl final of Opti-MEM before being added to the wells, which contained 500 µl of DMEM. Four hours later, 1.5 ml of DMEM was added to each well. Twenty-four hours post-transfection, the medium was changed and 50 nM of staurosporine was added where indicated. Cells were harvested 18 hours later and RNA was extracted using TriZOL (Invitrogen). Analysis of splicing profiles of the minigenes by RT-PCR was done as previously described (Garneau *et al.*, 2005). Visualization and analysis of amplified products for *Bcl-x* were done using the LabChip HT™ DNA assay on an automated microfluidic station (Caliper, Hopkinton, MA, USA).

RESULTS

Staurosporine modifies *Bcl-x* pre-mRNA splicing in 293 cells. In order to identify signaling pathways that impinge on the control of splice site selection of *Bcl-x*, we tested whether staurosporine, a general kinase inhibitor and inducer of apoptosis (Giuliano *et al.*, 2004; ten Dam *et al.*, 2000; Zhang *et al.*, 2004) could alter the relative abundance of the Bcl-x_S and Bcl-x_L splice isoforms in different cell lines. Staurosporine was applied to the adenovirus-transformed cell line 293 and six cell lines derived from a variety of cancers (U373, HeLa, SKOV-3, U-87, MCF7 and HCT 116). After 18 hours of incubation with 25 nM of staurosporine, the relative levels of endogenous Bcl-x_L and Bcl-x_S mRNAs were estimated by RT-PCR from total RNA (Fig. 1A). As expected, Bcl-x_L was the predominant form found in all mock-treated cells (Fig. 2A). Staurosporine had no significant effect on the relative abundance of the Bcl-x_L and Bcl-x_S isoforms, except in 293 cells where it drastically decreased the ratio of Bcl-x_L/Bcl-x_S.

The impact of staurosporine in 293 cells was dose-dependent (Fig. 2B). Because our staurosporine stock was dissolved in DMSO, and because DMSO can affect alternative splicing (Bolduc *et al.*, 2001), we tested the impact of DMSO on the relative abundance of *Bcl-x* isoforms. At its highest concentration, which was twice the concentration present in the 150 nM staurosporine solution, DMSO only affected the *Bcl-x* splice isoforms ratio by less than two-fold whereas staurosporine was usually shifting splicing by 10-fold (Fig. 2B, compare lane 8 with lane 7). The effect of staurosporine on *Bcl-x* splicing was visible after 12

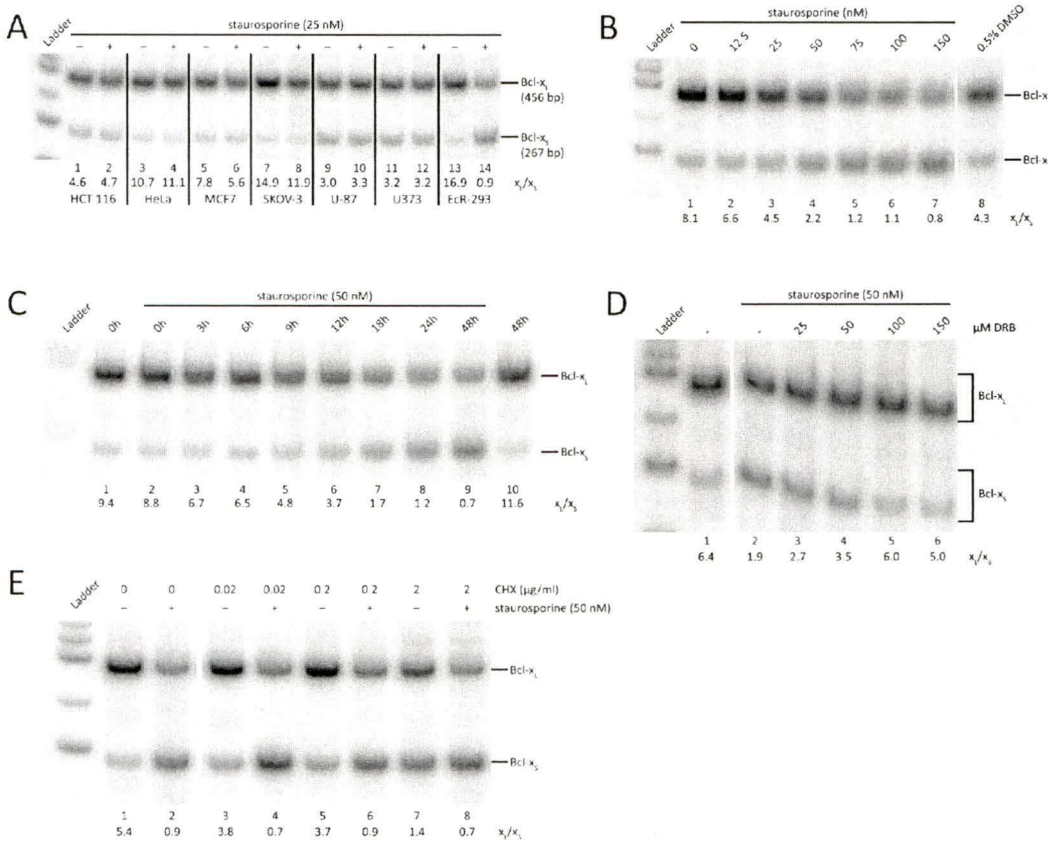

FIG. 2. Staurosporine affects *Bcl-x* pre-mRNA alternative splicing in 293 cells. (A) RT-PCR assays were performed on total RNA extracted from different human cell lines treated or not with staurosporine (25 nM). RT-PCR assays were also carried out to amplify portions of endogenous *Bcl-x* mRNAs when 293 cells were treated with increasing concentrations of staurosporine (panel B), for different incubation times at 25 nM of staurosporine (panel C) or when indicated concentrations of DRB (panel D) or cycloheximide (CHX, panel E) were added 1 hour prior to addition of 50 nM of staurosporine. A size ladder is shown on the left of all gels. The size of the different PCR products corresponding to the *Bcl-x* splice isoforms is indicated in panel A and the values of the Bcl-x_L/Bcl-x_S ratios are shown below lane numbers.

hours of incubation (Fig. 2C). Raising the concentration of staurosporine and/or increasing the incubation time did not change the *Bcl-x* isoform ratio in the cancer cell lines even though morphological changes characteristic of apoptosis (membrane blebbing, cell shrinkage, and formation of apoptotic bodies) were apparent in a majority of cells in all cell lines (data not shown). Thus, the signaling pathways that determine the relative abundance of *Bcl-x* mRNA isoforms in cancer cell lines are resistant to staurosporine.

Although the amplitude of the effect of staurosporine on the *Bcl-x* isoform ratio suggests that the drug might influence *Bcl-x* alternative splicing, it is possible that staurosporine differentially affects the stability of the *Bcl-x* mRNAs. To address this question, we tested the impact of staurosporine after inhibiting transcription elongation with increasing concentrations of DRB (Fig. 2D). Increasing concentrations of DRB antagonized the shift towards Bcl-x_S. This result indicates that the staurosporine-induced decrease in the Bcl-x_L/Bcl-x_S ratio requires active transcription, suggesting that differential stability of the *Bcl-x* mRNA isoforms does not make a major contribution to the shift induced by staurosporine.

To test whether the shift in the alternative splicing of *Bcl-x* requires de novo protein synthesis, we tested the impact of staurosporine in the presence of the protein synthesis inhibitor cycloheximide. When applied alone, cycloheximide stimulated Bcl-x_S splicing and this effect was dose-dependent (Fig. 2E, lanes 3, 5 and 7), possibly due to its activation of mitogen-activated and stress-activated kinases (Zinck *et al.*, 1995). Adding staurosporine one hour after the treatment with cycloheximide resulted in a marked increase in the Bcl-x_S product at all concentrations of cycloheximide (Fig. 2E, lanes 4, 6 and 8). Because cycloheximide did not antagonize the staurosporine-induced shift to Bcl-x_S, splicing modulation by staurosporine is likely achieved through the post-translational modification of splicing factors rather than through the expression of newly synthesized regulatory proteins.

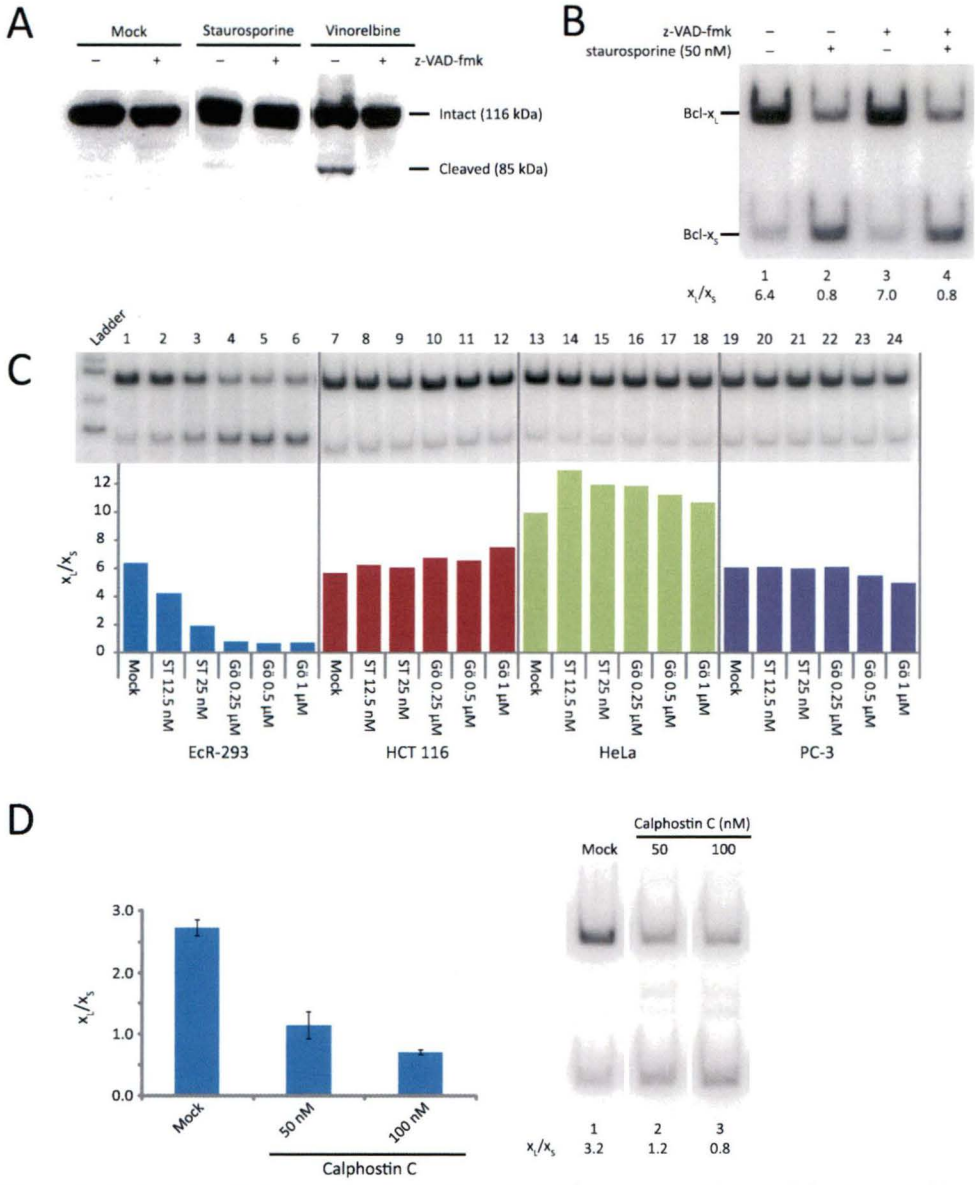

FIG. 3. Caspase-independent and PKC-dependent shifts in *Bcl-x* splicing. (A) Western blot performed to detect PARP cleavage induced by the different drugs in the absence or the presence of z-VAD-fmk. The position of the cleaved PARP product obtained with vinorelbine is shown. (B) RT-PCR assays were performed on total RNA from 293 cells treated for 18 hours with the caspase inhibitor z-VAD-fmk and/or staurosporine. The ratios of Bcl-x_L and Bcl-x_S products are indicated. (C) *Bcl-x* splicing profiles for different cell lines treated with the indicated concentrations of staurosporine (ST) and the specific PKC inhibitor Gö6976 (Gö). The graphs plot the Bcl-x_L/Bcl-x_S ratio. (D) Impact of the PKC inhibitor calphostin C on the endogenous *Bcl-x* splicing profile in 293 cells.

The effect of staurosporine on *Bcl-x* splicing is caspase-independent. Staurosporine can induce apoptosis through caspase-dependent or caspase–independent pathways (Belmokhtar *et al.*, 2001; Stepczynska *et al.*, 2001; Zhang *et al.*, 2004). Therefore, some of

the changes in the alternative splicing of *Bcl-x* may be due to caspase-dependent alterations in the integrity of splicing regulatory components. Indeed, many RNA binding proteins and spliceosome components can be cleaved during apoptosis (Casciola-Rosen *et al.*, 1994; Degen *et al.*, 2000; Fischer *et al.*, 2003). To test whether the shift in *Bcl-x* splicing was dependent on the activation of caspases, 293 cells were pre-treated with z-VAD-fmk, a general caspase inhibitor known to thwart apoptosis caused by staurosporine in a variety of cell lines (Giuliano *et al.*, 2004; Zhang *et al.*, 2004). Activation and inhibition of caspases by z-VAD-fmk was verified using the PARP cleavage assay (Lazebnik *et al.*, 1994). In comparison to vinorelbine, staurosporine did not elicit PARP cleavage and z-VAD-fmk prevented PARP cleavage induced by vinorelbine (Fig. 3A). Moreover, z-VAD-fmk had no basal effect on *Bcl-x* splicing and did not antagonize the staurosporine-mediated switch towards Bcl-x_S (Fig. 3B, lanes 3 and 4). Therefore, caspase activation does not contribute to the staurosporine-mediated change in *Bcl-x* splicing.

PKC inhibition shifts *Bcl-x* splicing. Staurosporine can inhibit a broad spectrum of kinases such as PKC, protein kinase G (PKG), protein kinase A (PKA), CaM kinase (CaMK), myosin light chain kinases and others (Ruegg et Burgess, 1989). However, it has a greater potency for protein kinase C (IC_{50} = 700 pM, Ki = 19 nM). To investigate whether staurosporine might act on *Bcl-x* splicing by inactivating PKC, we tested the specific PKC inhibitor Gö6976 (IC_{50} = 7.9 nM, Ki = 2.8 nM) (Koivunen *et al.*, 2004; Martiny-Baron *et al.*, 1993). The incubation of 293 cells for 18 hours with Gö6976 promoted an increase in the Bcl-x_S product, as seen with staurosporine (Fig. 3C, lanes 1-6). Inhibiting PKA or PKG using H-89 (45 nM, K_i = 48 nM) or

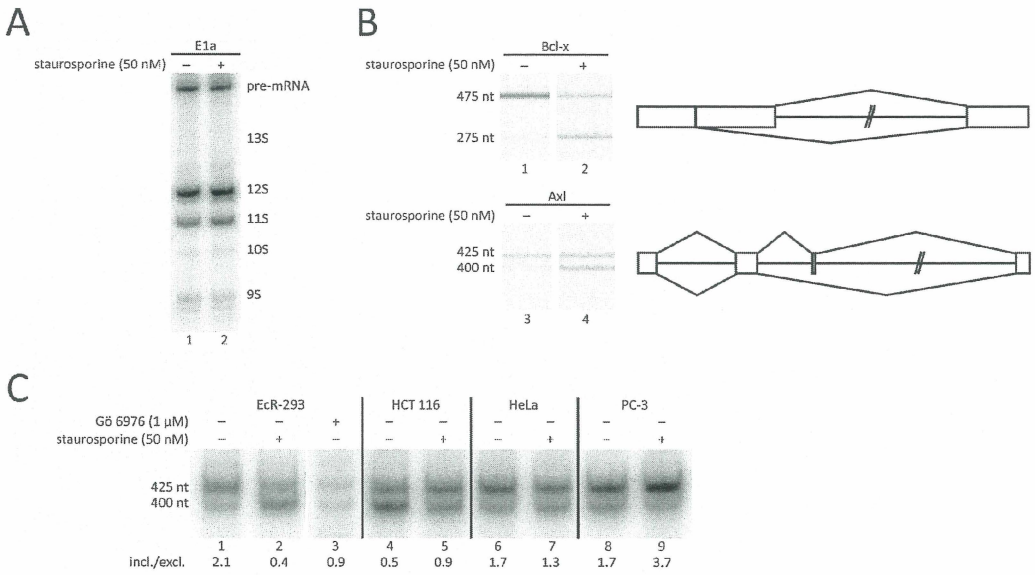

FIG. 4. PKC-dependent alternative splicing of *Axl*. (A) A RT-PCR assay was carried out to amplify endogenous adenovirus E1a products in 293 cells treated or not with 50 nM of staurosporine. (B) RT-PCR assays were designed to amplify splicing products from *Bcl-x* and *Axl* in mock- or staurosporine-treated 293 cells. The sizes of the PCR products are indicated as well as the structure of the *Bcl-x* and *Axl* alternative splicing units. (C) Different cell lines were treated with staurosporine or Gö6976. RT-PCR assays were performed to amplify *Axl* splicing products. The inclusion/exclusion ratios are shown below lane numbers.

DT-3 (45 nM, K_i = 25 nM), respectively, only minimally increased the production of Bcl-x_S in 293 cells (data not shown). Thus, the impact of staurosporine on the alternative splicing of *Bcl-x* in 293 cells is likely to be mediated mostly through the PKC pathway. Other observations are relevant to the role of PKC in *Bcl-x* splicing. First, DMSO can induce PKC-mediated apoptosis (Ginestier-Verne *et al.*, 1996), possibly explaining the small effect of DMSO on *Bcl-x* splicing (Fig. 2B, lane 8). Second, calphostin C, a highly specific inhibitor of PKC (IC_{50} = 50 nM) (Kobayashi *et al.*, 1989) also increased the production of Bcl-x_S in 293 cells (Fig. 3D). Third, tamoxifen, which has been reported to inhibit PKC (O'Brian *et al.*, 1985), increased the relative level of Bcl-x_S in 293 cells (not shown). While calphostin C can inhibit several members of the PKC family, Gö6976 is reported to inhibit PKCα, PKCβ and PKCμ (now known as PKD), but not PKCγ, PKCδ, PKCε and PKCξ (Koivunen *et al.*, 2004; Zhang *et al.*, 2005). A specific PKCβ inhibitor did not affect *Bcl-x* splicing in 293 cells, nor did Gö6983, an

inhibitor of PKCα but not of PKD (Zhang *et al.*, 2005) (data not shown). Knocking down PKCα by RNA interference did not affect Bcl-x splicing and did not antagonize the shifting activity of staurosporine (data not shown). While the above results might point to PKD as the PKC enzyme involved in *Bcl-x* splicing control, some studies indicate that PKD activity is not affected by staurosporine (Zhang *et al.*, 2005). To identify which PKC enzyme affects *Bcl-x* splicing in 293 cells, we have initiated a systematic RNA interference approach targeting individual PKC enzymes.

Modulation of *Bcl-x* and *Axl* splicing by staurosporine does not occur in cancer cell lines. Notably, staurosporine and Gö6976 did not improve the production of Bcl-x_S in the cancer cell lines HCT 116, HeLa and PC-3 (Fig. 3C, lanes 7-24). The PKC pathway is known to be abnormally regulated in many cancer cell lines (Koivunen *et al.*, 2006), which may explain why the alternative splicing of *Bcl-x* in this selection of cancer cell lines is not affected.

To identify other alternative splicing events that might be controlled by PKC, we tested the impact of staurosporine on other pre-mRNA substrates expressed in 293 cells. First, we tested the production of isoforms produced from the adenovirus E1a pre-mRNA, a gene whose expression contributes to the transformed state of 293 cells. Like *Bcl-x*, E1a uses alternative 5' splice site usage to produce a variety of isoforms (13S, 12S and 9S mRNAs). Staurosporine did not affect the alternative splicing of E1a (Fig. 4A). Thus, the impact of staurosporine appears not to involve a factor that controls alternative 5' splice site selection in a global manner.

Next, we tested the impact of staurosporine on the production of mRNAs isoforms produced from a selection of apoptotic genes expressed in 293 cells. While most of the splicing units tested were not affected by staurosporine, the alternative splicing of the receptor tyrosine kinase *Axl* was significantly affected (Fig. 4B, lanes 3 and 4). The skipping of an alternative exon in *Axl* was also stimulated by the PKC inhibitor Gö6976 (Fig. 4C, lane 3). Although aberrant expression of *Axl* has been linked to cancer (Green *et al.*, 2006; van Ginkel *et al.*, 2004), the function of the short isoform whose abundance is enhanced by staurosporine is currently unknown. Thus, the PKC pathway likely controls the alternative splicing of a subset of pre-mRNAs in 293 cells. As was the case with *Bcl-x*, staurosporine did not stimulate exon skipping of *Axl* in the HCT116, HeLa and PC-3 cancer cell lines (Fig. 4C).

Staurosporine mediates its activity through an element in *Bcl-x* exon 2. If the PKC-dependent effect of staurosporine on *Bcl-x* splicing is specific and not due to general splicing deregulation, this effect may be exerted through a protein that binds to a sequence element in the *Bcl-x* pre-mRNA. To identify regions in the *Bcl-x* pre-mRNA that may be necessary for the activity of staurosporine, we compared the splicing ratios of transcripts derived from *Bcl-x* minigenes (Fig. 5A). X2 contains exon 2 and 1.2 kb of downstream intron sequence with exon 3 and neighboring upstream intron sequences (Garneau *et al.*, 2005). X2.13 is identical to X2 except that it lacks the first half of exon 2. Following transfection of the *Bcl-x* minigenes in 293 cells, RT-PCR analysis indicated that transcripts from X2 were spliced more efficiently to the Bcl-x_L 5′ splice site than those expressed from X2.13 (Fig. 5B, compare lane 1 with lane 3 and accompanying graph in right panel). Thus, the 361 nt region forming the first half of exon 2, dubbed SB1, behaves as an inhibitor of the Bcl-x_S 5′ splice site. When the analysis was performed in transfected cells treated with staurosporine, we observed a shift

towards Bcl-x_S for transcripts derived from X2, thereby reproducing the impact of staurosporine on endogenous *Bcl-x* transcripts (Fig. 5B, lane 2). In contrast, the same treatment had a much-reduced impact on the ratio of isoforms produced from X2.13 (lane 4). These results indicate that staurosporine can lift the basal repression of the Bcl-x_S 5′ splice site that is provided by SB1. We also tested mutated derivatives of X2 and X2.13 (X2-W and X2.13-W; Fig. 5A) that produce more of the Bcl-x_L isoform. In these cases, the repressor activity of SB1 was also apparent (Fig. 5B, compare lane 5 and lane 7, and accompanying graph on right panel), and the production of Bcl-x_S was stimulated by staurosporine in an SB1-dependent manner (Fig. 5B, lanes 6 and 8).

Although staurosporine did not promote an increase in the level of the Bcl-x_S mRNA in cancer cells (Fig. 2A), we transfected the X2 and X2.13 minigenes in HeLa cells to ask if the SB1 element had any activity in this cell line. Notably, the use of the Bcl-x_S 5′ splice site was stronger in the absence of the SB1 element (Fig. 5C), confirming a previous observation made in HeLa cells (Garneau *et al.*, 2005), and indicating that the SB1 element is actively repressing the x_S donor splice site. This result suggests that the SB1 element functions in cancer cells but that staurosporine is unable to lift the repression.

To confirm the role of SB1 and the neutralization of its activity by staurosporine, we asked whether portions of SB1 were capable of directing splicing decisions in different contexts. As shown in Figure 6A, we inserted three partially overlapping portions of SB1 into the first exon of Dup51, a model splicing unit derived from duplicated human ?-globin regions (Dominski et Kole, 1991). Following transfection in 293 cells and RT-PCR analysis, we observed that all portions of SB1 enhanced the skipping of the central exon (Fig. 6B);

fragment C (159 nt) having the strongest effect (lane 7). When transfected cells were treated with staurosporine, the alternative splicing of Dup51 and of derivatives containing fragments A and B was not significantly affected (Fig. 6B, lanes 2, 4 and 6). In contrast, staurosporine neutralized the exon skipping activity of fragment C (lane 8). We also tested the activity of

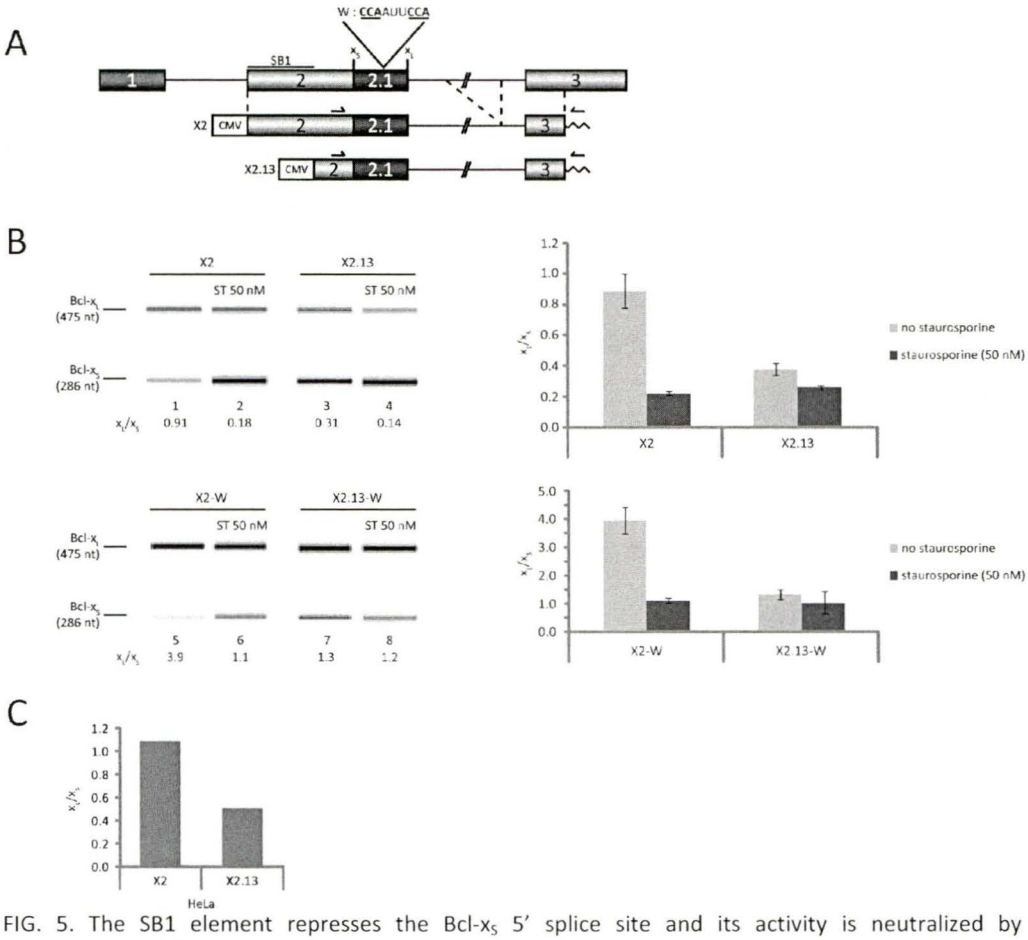

FIG. 5. The SB1 element represses the Bcl-x_S 5′ splice site and its activity is neutralized by staurosporine. (A) Diagram representing the SB1 element on the *Bcl-x* gene, and the structure of minigenes X2 and X2.13. Derivatives of X2 and X2.13 carrying mutations (two GGU mutated to CCA) that improve Bcl-x_L production were also used (X2-W and X2.13-W). The position of the primers used to amplify mRNA products from these minigenes is indicated. (B) The *Bcl-x* splicing products were monitored by RT-PCR amplification from total RNA isolated from mock- or staurosporine-treated 293 cells transfected with minigenes. The analysis of amplified products was done on an automated microfluidic station and electropherograms are shown. The size of the amplified *Bcl-x* products is indicated as well as the x_L/x_S ratios. The graphs on the right represent the quantitated x_L/x_S average ratios from another set of experiments performed with the X2.13, X2, X2.13-W and X2-W minigenes. Standard deviations are provided. (C) Graph representing the x_L/x_S ratios obtained following transfections with the X2 and X2.13 minigenes in HeLa cells.

portions B and C of SB1 when inserted at an upstream position in an adenovirus E1a minigene (Yang *et al.*, 1994). In this case, only fragment B influenced alternative splicing by improving the inclusion of a central exon that produces the 10S isoform (Fig. 6C, lane 3). Staurosporine enhanced the inclusion of this exon in a fragment B-dependent manner (lane 4). Thus, fragment C displayed splicing modulatory activity in Dup51 and mediated the staurosporine splicing shift, while this behavior was a characteristic of fragment B in E1a. Although fragments B and C are overlapping, our results suggest that the SB1 element is complex and that the activity of distinct portions of SB1 is sensitive to the context in which they are tested. Nevertheless, it is clear that portions of SB1 can modulate splice site selection in an heterologous manner, and that responsiveness to staurosporine can be transfered to other pre-mRNAs. Thus, our results suggest that SB1 is bound by factors whose activity is connected to the PKC pathway.

DISCUSSION

PKC-dependent control of *Bcl-x* splicing in 293 cells. As can be expected for a gene that produces isoforms with antagonistic functions in apoptosis, the alternative splicing of *Bcl-x* is highly regulated and several sequence elements contribute to its splicing control. Among these are sequences that may couple splicing decisions with the transduction of signals initiated by growth factors and ceramide (Li *et al.*, 2004; Massiello *et al.*, 2004). Although staurosporine can downregulate Bcl-x_L levels in a few cell lines, a corresponding increase in Bcl-x_S was not observed previously (Giuliano *et al.*, 2004). Our study indicates that

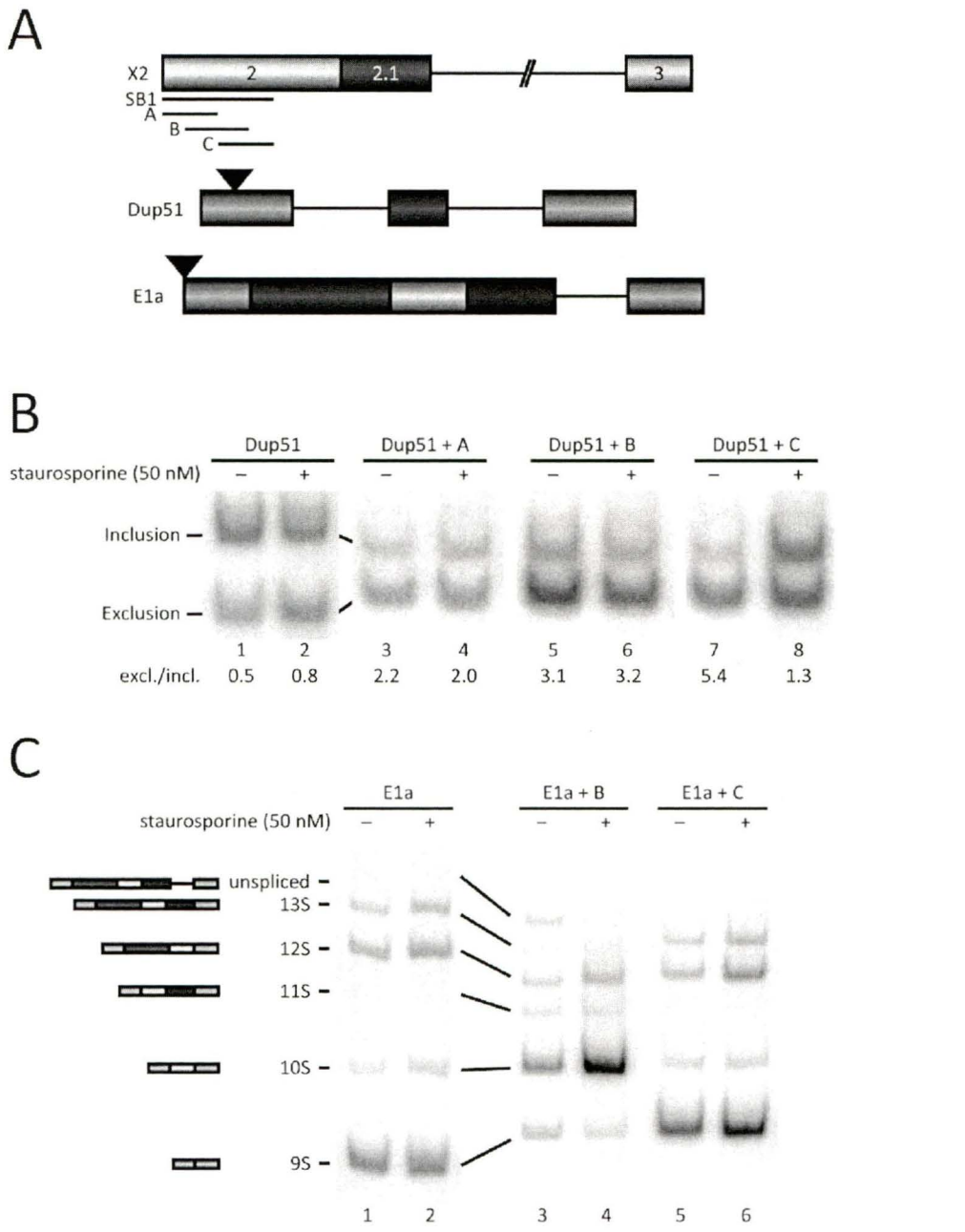

FIG. 6. Portions of SB1 can modulate splicing in heterologous pre-mRNAs. (A) Diagrams of the *Bcl-x* X2, the globin-derived Dup51 and the adenovirus E1a pre-mRNAs indicating the portions of SB1 that were inserted into Dup51 and E1a. (B) RT-PCR assays were performed to amplify products derived from the minigenes transfected in 293 cells that were treated or not with staurosporine. Values for the exclusion/inclusion ratio are shown below lane numbers. (C) RT-PCR assays were carried out to amplify products derived from the E1a minigene transfected in 293 cells that were treated or not with staurosporine. The position and structure of the various splicing products are indicated.

staurosporine stimulates the production of the pro-apoptotic Bcl-x_S isoform in 293 cells. Because a pre-treatment with the transcriptional inhibitor DRB prevents the increase in Bcl-x_S seen with staurosporine, this drug appears to act on the alternative splicing of *Bcl-x* rather than on the differential stabilities of mRNA isoforms. Moreover, the impact of staurosporine is not caused through protein cleavage since the incubation with a general inhibitor of caspases did not prevent the shift in *Bcl-x* splicing.

The fact that the translational inhibitor cycloheximide did not antagonize the staurosporine-induced splicing shift suggests that signaling events are responsible for the impact of staurosporine on *Bcl-x* splicing. Staurosporine is a known PKC inhibitor. Consistent with the possibility that staurosporine alters *Bcl-x* splicing in 293 cells through repression of PKC, we observed that the PKC□/PKC□/PKCμ inhibitor Gö6976 and the highly specific PKC inhibitor calphostatin C can mimic the staurosporine-mediated increase in Bcl-x_S in 293 cells. This situation is similar to the PKC/Ras-induced shift in alternative splicing of CD44 (Konig *et al.*, 1998), but contrasts with the alternative splicing of CD45 where the PKC/Ras-dependent splicing switch is blocked by cycloheximide, indicating that one or several proteins must be made following activation (Lynch et Weiss, 2000).

The involvement of PKC in the control of *Bcl-x* splicing is not totally surprising. On the one hand, suppression of PKC is associated with the induction of apoptosis that at least in some cases is accompanied by a reduction in the expression of Bcl-x_L (Giuliano *et al.*, 2004; Hsieh *et al.*, 2003; Leirdal et Sioud, 1999; Savickiene *et al.*, 1999). On the other hand, ceramide, which promotes Bcl-x_S usage in A549 cells, is known to repress PKC□ (Lee *et al.*, 1996) and to induce the ceramide-activated protein phosphatase PP1 (Chalfant *et al.*, 2002). However, the

elements that have been associated with the response to ceramide are distinct from SB1 (Massiello *et al.*, 2004). Nevertheless, because the role of SB1 in the response to ceramide has not been examined, it is possible that part of the PKC-dependent impact of ceramide is exerted through SB1.

An exon element mediates the PKC-dependent repression of Bcl-x_S. We have linked the effect of staurosporine on *Bcl-x* splicing to a 361 nt element (SB1) situated 187 nt upstream of the Bcl-x_S 5′ splice site. The SB1 element displays basal repressor activity because its removal stimulates the use of the Bcl-x_S 5′ splice site in transfection assays performed in 293 and in HeLa cells. Thus, 293 and HeLa cells likely contain a factor that mediates the repressor activity of the SB1 element. This interpretation is consistent with the observation that transfecting large amounts of *Bcl-x* plasmid in 293 cells increased the relative level of Bcl-x_S and antagonized our ability to detect the SB1-dependent effect (data not shown). However, as suggested by testing SB1 fragments in a heterologous context, the mechanism of action of SB1 is probably more complex because exon skipping in Dup51 was promoted by different portions of SB1 whereas a subregion of SB1 (fragment B) elicited exon inclusion in E1a.

In the absence of SB1, splicing to Bcl-x_S was stronger and was not further stimulated by staurosporine. Thus, staurosporine appears to neutralize the repressor activity mediated by the SB1 element in 293 cells. In a heterologous context, the portions of SB1 that conferred response to staurosporine stimulated the inclusion of downstream exons, a situation that is difficult to reconcile with a simple derepression model. Moreover, the portions of SB1 that conferred responsiveness to staurosporine varied with different pre-mRNAs. A more

exhaustive mutational analysis of the SB1 element and the identification of the factors that bind to it should help us dissect this apparently complex mechanism of splicing control.

Given the role of PKC in the activity of SB1 and the lack of a requirement for new protein synthesis, our results suggest that a phosphorylated protein may be essential for the activity of SB1. The inhibition of PKC may shift the equilibrium towards a dephosphorylated form that may not be binding to SB1 or that may be incapable of sustaining interactions with other proteins involved in the repression of the 5' splice site Bcl-x_S. We have been unable to reproduce in vitro the impact of SB1 on *Bcl-x* splicing using 293 extracts, a situation that may also point to a particularly labile phosphorylation event that is lost during extract preparation. The identity of this *trans*-acting factor therefore remains to be determined. Phosphorylation-dependent binding has been observed for many RNA binding proteins including SR proteins (Guil *et al.*, 2006; Huang *et al.*, 2004; Lai *et al.*, 2003; Prasad *et al.*, 1999; Tacke *et al.*, 1997). Interestingly, PKC can interact with PSF, hnRNP A3, p68 RNA helicase and hnRNP L (Rosenberger *et al.*, 2002). The alternative splicing of CD45 during T-cell activation has also implicated both PKC and hnRNP L, the latter protein interacting directly with sequences in the regulated exons (Lynch et Weiss, 2000; Rothrock *et al.*, 2005). Knocking down hnRNP L in HeLa cells did not affect *Bcl-x* splicing (data not shown). Sam68 has recently been shown to improve the production of the Bcl-x_S isoform in HEK293 cells (Paronetto *et al.*, 2007). Although the binding site for Sam68 in *Bcl-x* has not been mapped, Sam68 may counteract the activity of the repressor that binds to SB1.

We have observed that staurosporine and Gö6976 can also affect the alternative splicing of *Axl*, a gene encoding a protein kinase receptor whose expression is altered in cancer cells

(Holland *et al.*, 2005; Shieh *et al.*, 2005). The larger isoform of *Axl* protects cells against apoptosis when bound to its receptor Gas6 (Lee *et al.*, 1999; Shankar *et al.*, 2006), but the function of the smaller isoform is unknown. Thus, the PKC signaling pathway is involved in modulating the splicing of several pre-mRNAs, but it is unclear whether all PKC-dependent splicing shifts converge on the same splicing regulatory factor(s). Comparing the sequences shared by fragments B and C of exon 2 in *Bcl-x* with the equivalent region in *Axl* revealed common stretches of CU-rich sequences. A mutational approach combined with swapping experiments should help determine if the staurosporine-induced splicing shift of *Bcl-x* and *Axl* is mediated by functionally similar elements.

Altered PKC pathway in cancer cells. Of the eight cell lines that were treated with staurosporine, a shift towards the pro-apoptotic Bcl-x_S isoform was observed only in 293 cells. Likewise, the alternative splicing of *Axl* was modulated by staurosporine in 293 cells but not in the cancer cell lines. It is unlikely that this selectivity reflects an inability of the drug to reach sufficiently high intracellular concentrations in cancer cells since staurosporine induced a phenotype characteristic of apoptosis in all cell lines. Moreover, the PKC inhibitor Gö6976 was also unable to promote a splicing switch in the cancer cell lines. Thus, the PKC pathway in the cancer cell lines tested is either inactive or is resistant to the action of inhibitors. We favor the latter explanation because activation of the PKC□/PKC□ isozymes has been linked to malignancy (Koivunen *et al.*, 2006). In addition, the SB1 element that represses the Bcl-x_S 5′ splice site is functional in HeLa cells. The molecular basis for the resistance of the PKC pathway to staurosporine and to Gö6976 remains unclear. Different PKC isoforms and isozymes may be expressed in cancer cells (Aaltonen *et al.*, 2006; Langzam *et al.*, 2001; Varga *et al.*, 2004). These PKC variants may be less sensitive to inhibitors or they

may not phosphorylate the proteins that relay repression through the SB1 element in 293 cells. Alternatively, PKC activity may be more susceptible to inhibition in 293 cells. Consistent with this view, the expression of the adenovirus E1a gene can promote a relocalization of PKC to membranes (Shiroki *et al.*, 1992).

Based on these observations, we are now testing the impact of a battery of chemotherapeutic agents on the alternative splicing of *Bcl-x* and other genes in a variety of cell lines. In addition to help uncover networks of splicing regulation, this approach may reveal cancer-specific differences in abilities to couple signaling events with splicing decisions.

ACKNOWLEDGEMENTS

We thank Catherine Desrosiers for the production of various polymerases.

This study was supported initially by a grant from National Cancer Institute of Canada and later by a grant from the Canadian Institute of Health Research. We thank Uli Froehlich and the Laboratory of Functional Genomics of the Université de Sherbrooke for their helpful contribution. B.C. is a Canada Research Chair in Functional Genomics.

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Chapitre II

Revil, T., Pelletier, J., Toutant, J., et Chabot, B. 2008. Heterogeneous nuclear ribonucleoprotein K represses the production of the pro-apoptotic Bcl-xS splice isoform. Soumis à *Journal of Biological Chemistry*.

Résumé

Le pré-ARNm de Bcl-x est épissé de façon alternative pour produire les isoformes Bcl-x_L, anti-apoptotique, et Bcl-x_S, pro-apoptotique. En effectuant des mutations par délétions sur un mini-gène de Bcl-x humain, nous avons identifié un nouvel élément exonique qui contrôle l'utilisation du site d'épissage 5' de Bcl-x_S. La partie proximale de cet élément agit comme un répresseur et est située en aval d'un activateur. Des analyses additionnelles par mutations ponctuelles ont procuré une carte topographique des activités régulatrices, indiquant une transition rapide entre les séquences activatrices et inhibitrices. Des portions de l'activateur peuvent fonctionner lorsque placées dans une autre unité d'épissage alternatif. Des expériences de chromatographie et d'immunoprécipitation indiquent que l'élément inhibiteur interagit avec hnRNP K, ce qui est cohérent avec la présence potentielle de sites de haute affinité pour cette protéine. Finalement, la baisse de la concentration de hnRNP K par interférence à l'ARN a favorisé la formation de Bcl-x_S, un effet observé seulement lorsque les séquences liées par hnRNP K étaient présentes. Nos résultats démontrent donc un rôle clair pour hnRNP K dans l'inhibition de la production de l'isoforme pro-apoptotique Bcl-x_S.

J'ai réalisé toutes les expériences dans cet article et j'ai contribué à l'écriture de cet article. Jordan Pelletier a effectué plusieurs mutations ponctuelles dans les régions B1u et Johanne Toutant était responsable des manipulations cellulaires.

Heterogeneous Nuclear Ribonucleoprotein K Represses the Production of the Pro-apoptotic Bcl-x_S Splice Isoform

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Running head: hnRNP K represses the Bcl-x_S isoform

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ABSTRACT

The Bcl-x pre-mRNA is alternatively spliced to produce the anti-apoptotic Bcl-x_L and the pro-apoptotic Bcl-x_S isoforms. By performing deletion mutagenesis on a human Bcl-x minigene,

we have identified a novel exonic element that controls the use of the 5' splice site of Bcl-x_S. The proximal portion of this element acts as a repressor and is located downstream of an enhancer. Further mutational analysis provided a detailed topological map of the regulatory activities revealing a sharp transition between enhancer and repressor sequences. Portions of the enhancer can function when transplanted into another alternative splicing unit. Chromatography and immuno-precipitation assays indicate that the silencer element interacts with hnRNP K, consistent with the presence of putative high-affinity sites for this protein. Finally, downregulation of hnRNP K by RNA interference enhanced splicing to Bcl-x_S, an effect seen only when the sequences bound by hnRNP K are present. Our results therefore document a clear role for hnRNP K in preventing the production of the pro-apoptotic Bcl-x_S splice isoform.

INTRODUCTION

Alternative splicing is a major mechanism used to augment the number of proteins encoded by the genome. It is estimated that as many as 97% of multi-exon pre-mRNAs undergo alternative splicing (Pan *et al.*, 2008; Wang *et al.*, 2008). Disruption of alternative splicing by mutating important regulatory sequences or by altering the expression or activity of proteins controlling splice site selection has been linked with different diseases including cancer (Baralle et Baralle, 2005; Garcia-Blanco *et al.*, 2004; Shkreta *et al.*, 2008; Venables, 2006; Wang et Cooper, 2007). Apoptosis is an important and complex cellular program involved in development and differentiation in higher organisms (Baehrecke, 2002; Hipfner et Cohen, 2004). However, its aberrant control often contributes to cancer development and the resistance of cancer cells to drug therapy (Fulda et Debatin, 2006; Green et Evan, 2002; Tsuruo *et al.*, 2003; Viktorsson *et al.*, 2005).

Genes implicated in apoptosis are often alternatively spliced to produce protein isoforms with distinct or even antagonistic activities (Akgul *et al.*, 2004; Schwerk et Schulze-Osthoff, 2005). A good example is the apoptotic regulator Bcl-x, which is alternatively spliced to produce two major isoforms, the anti-apoptotic Bcl-xL protein and the shorter pro-apoptotic Bcl-xS isoform (Boise *et al.*, 1993). This alternative splicing decision involves a competition between two 5' splice sites; the use of the downstream site creates Bcl-xL, while the use of the upstream one produces Bcl-xS (Fig. 1A). Bcl-xL is always the predominant form in cancer cells and overexpressing it can confer resistance to chemotherapeutic agents (Castilla *et al.*, 2006; Espana *et al.*, 2004; Lebedeva *et al.*, 2000; Linden *et al.*, 2004; Wang *et al.*, 2006; Watanabe *et al.*, 2002). On the other hand, overexpression of the pro-apoptotic Bcl-xS isoform enhances sensitivity to the topoisomerase inhibitor etoposide and to taxol in a breast cancer cell line, while triggering apoptosis in melanoma cell lines (Hossini *et al.*, 2003; Sumantran *et al.*, 1995). Using antisense technologies to improve the production of the Bcl-xS splice variant can also induce apoptosis in cancer cells (Mercatante *et al.*, 2001; Mercatante *et al.*, 2002; Taylor *et al.*, 1999; Wilusz *et al.*, 2005).

Alternative splicing is regulated by different proteins bound to sequence elements near splice sites. A variety of mechanisms is used to achieve regulation. Some splicing factors act by recruiting or inhibiting the binding of different components of the spliceosome. Others may change the conformation of the pre-mRNA to mask a splice site or to bring a pair of splice sites in closer proximity (Amir-Ahmady *et al.*, 2005; Martinez-Contreras *et al.*, 2006).

Although individual factors can have a strong and specific effect on splicing decisions, alternative splicing often relies on a combination of factors to determine the appropriate levels of isoforms. The implication of multiple proteins likely provides additional levels of regulation that helps tune splicing control to a variety of stresses, environmental cues and growth conditions. In several cases, the interaction of regulatory factors can be antagonistic. For example, in the Drosophila male-specific-lethal-2 (msl-2) pre-mRNA, recruitment of SXL to a uridine-rich region interferes with the binding of TIA-1 which is necessary for efficient U1 snRNP recruitment at the 5' splice site (Forch *et al.*, 2001). On the same pre-mRNA, SXL also diminishes U2AF recognition of the polypyrimidine tract at the 3' splice site. TIA proteins bound to a U-rich element on the avian myosin phosphatase targeting subunit-1 (MYPT1) pre-mRNA repress the binding of PTB (Shukla *et al.*, 2005). PTB can also reduce the recruitment of ETR-3 to intronic elements near exon 5 of cardiac troponin T (cTNT) (Charlet *et al.*, 2002). In neurons, the binding of PTB to the introns surrounding the N1 exon of c-src is antagonized by nPTB protein, promoting exon inclusion. On the hnRNP A1 pre-mRNA, PTB diminishes the binding of SRp30c to the intronic CE9 element, reducing the inhibition by this protein on the use of the downstream 3' splice site (Paradis *et al.*, 2007). SC35 and hnRNP A1 have partially overlapping binding sites on the human immunodeficiency virus 1 (HIV-1) tat exon 2. Preferential binding of SC35 enhances the inclusion of the exon, whereas hnRNP A1, by reducing SC35 binding, increases exclusion (Zahler *et al.*, 2004). Thus, the competition provided by an overlapping or a closely abutting pair of enhancer/silencer represents a simple and frequent mechanism of splicing control.

The regulation of Bcl-x alternative splicing has received some attention in recent years leading to the discovery of several cis-acting elements and a few trans-acting control factors

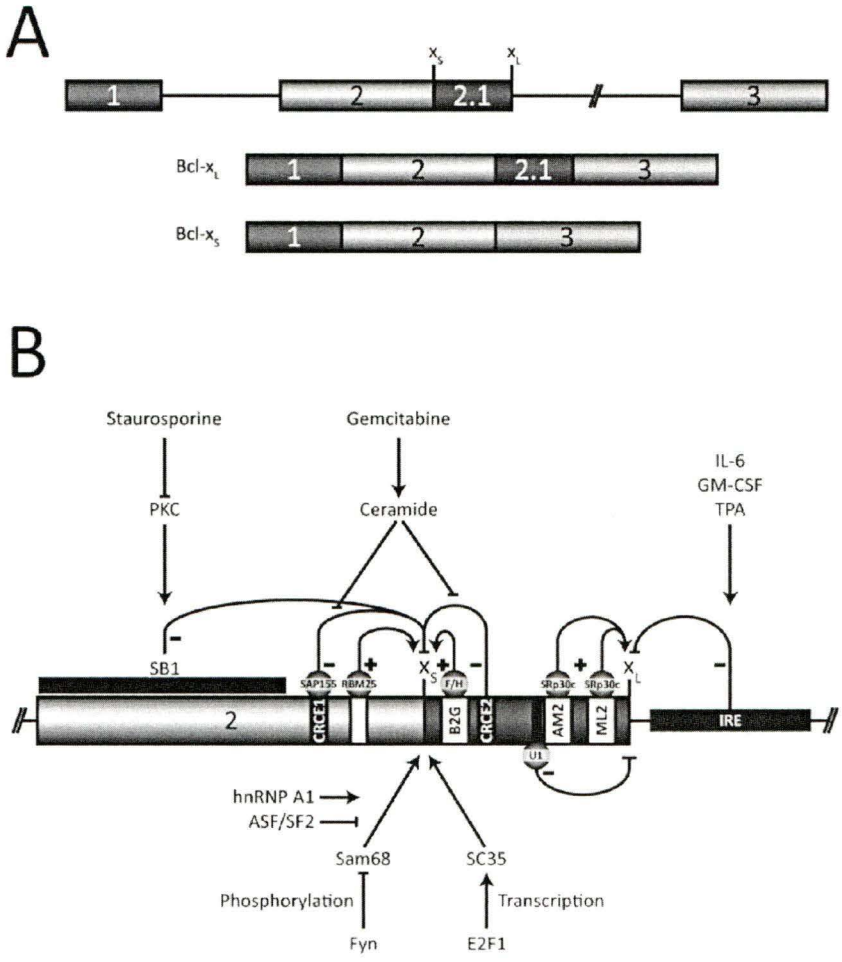

Fig. 1. *A.* The alternative splicing of Bcl-x produces two major isoforms, Bcl-x_L and Bcl-x_S. *B.* Regulation of bcl-x alternative splicing. The enhancer elements are shown as white boxes while the repressors are black. The pointed and flat arrows indicate positive and negative regulation, respectively. Protein kinase C inhibition relieves repression caused by the SB1 element on the Bcl-x_S splice site (37). The repressor elements CRCE1, recognized by SAP155, and CRCE2 mediate the production of Bcl-x_S by the newly synthesized ceramide induced by gemcitabine in A549 cells (39,40). hnRNP F/H bind to the B2G element to enhance the production of the Bcl-x_S isoform (42). RBM25, through an element located upstream of the Bcl-x_S splice site, can also augment its use (45). A large intronic region (IRE) mediates the Bcl-x_L increase caused by IL-6, GM-CSF and TPA (36). Finally, the B3 region also enhances Bcl-x_L formation through the binding of SRp30c to AM2 and ML2, and the U1 snRNP to two cryptic 5' splice sites (43).

(Fig. 1*B*). Intronic regions downstream from the Bcl-x_L 5' splice site have been implicated as mediating signals from cytokines such as interleukin-6 and granulocyte-macrophage colony-stimulating factor (Li *et al.*, 2004b). In addition, we have reported that an element located 187 nt upstream of the Bcl-xS splice site mediates a protein kinase C-dependent signal that represses splicing to the Bcl-xS donor site (Revil *et al.*, 2007). On the other hand, ceramide

enhances the use of the Bcl-xS 5' splice site by lifting the repression mediated by two other elements (Chalfant *et al.*, 2002; Massiello *et al.*, 2004). The activity of one of these apparently involves SAP155 (Massiello *et al.*, 2006). The RNA binding protein Sam68, under the control of the tyrosine kinase Fyn, can also increase the production of Bcl-xS in cooperation with hnRNP A1 (Paronetto *et al.*, 2007), and this effect is inhibited by overexpression of ASF/SF2. The Bcl-x sequences bound by the above factors remain to be identified. We also uncovered enhancer elements for Bcl-xS and Bcl-xL. hnRNP F and H binds downstream of the Bcl-xS 5' splice site to stimulate splicing to that site (Garneau *et al.*, 2005). Enhancement of Bcl-xL is conferred by SRp30c which binds upstream of the 5' splice site to antagonize the repressor activity of pseudo 5' splice sites (Cloutier *et al.*, 2008). Recently, the SR protein SC35 was shown to increase the production of Bcl-xS (Merdzhanova *et al.*, 2008). Finally, the binding of RBM25 to a sequence element upstream of the Bcl-xS 5' splice site stimulated its use, possibly by recruiting U1 snRNP through its interaction with the U1-associated protein hLuc7A (Zhou *et al.*, 2008). Thus, the region located in between the two competing 5' splice sites of Bcl-x is densely populated by splicing control elements.

In the current study, we have pursued our characterization of Bcl-x splicing control by examining the contribution of sequences directly upstream of the Bcl-x_S donor site. Our mutational approach identified a region containing flanking enhancer and silencer activities. The activity of the repressor portion is mediated by hnRNP K, which makes this protein a potential anti-apoptotic regulator.

EXPERIMENTAL PROCEDURES

Cell culture- The 293 cells used in this study were the EcR-293 cell line (Invitrogen). EcR-293 and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% glutamine. PC-3 cells were maintained in Ham's F12 medium containing 10% fetal bovine serum and 1% glutamine.

Plasmid construction- The Bcl-x minigene X2.13 was constructed as described previously (Garneau *et al.*, 2005). The ΔB1 deletion was created by amplifying the X2.13 with primers XS+BsmI (ATATAGGGCATTCCTTTGAACAGGT) and Bcl-x AccI (ATCTCCTTGTCTACGCTT) using Pfu polymerase. The resulting insert was digested with BsmI and AccI and inserted in the S2.13 mini-gene digested with the same enzymes. The resulting construction was inserted in pcDNA3.1+ as previously described (Garneau *et al.*, 2005). To construct ΔB1d, a first fragment of X2.13 was amplified with Pfu using oligos B1down25 (TACCGGCGGGCATT-CTCACCCCAGGGACAG) and Human-4 (ATGCCTGATCTCTGAAGC-ACAG). A second fragment was amplified using B1down25-B (GAATGCCCGCCGGTACCGCAG) and RT-1 (GAACCCACTG-CTTACTGGCT). Both fragments were purified on gel and extracted (Qiagen), then mixed in equimolar quantities and amplified with Pfu using oligos RT-1 and Human-4. The resulting product was digested with NheI and HpaI, purified on agarose gel and ligated in X2.13 previously digested with the same enzymes. The same technique was used to obtain ΔB1u (oligos B1up25 (ACTACCTGTTCAAAGTGTGGAGCTGGGATG) with Human-4 and B1up25-B (CTTTGAACAGGTAGTGAATGA) with RT-1), ΔB1AG (oligos B1AGdown25 (GGAGGCAGGCGAA-GTGACCTGACA) with Human-4 and B1ACGdown25-B (TCGCCTGCCTCCCTC) with RT-1) and ΔB1AC (oligos B1ACdown25 (GGAGGCAGGCGATCACCCCAGGGA) with Human-4 and

B1ACGdown25-B with RT-1). Point mutations were obtained by Pfu amplification using the same overlapping primer technique. To insert the elements in minigene 45, we hybridized oligos representing the elements using an equimolar ratio of DNA oligomers for the sense and antisense strands, incubated in 1X One-Phor-All buffer (GE Healthcare) at 100°C for 10 min and then slowly cooling down at 21°C during 2 hours. These mixtures were then ligated in minigene 45 previously cut with BseRI and blunted with Klenow. All constructs were verified by digestion and sequencing.

Transfection of plasmids or siRNA, and RNA extraction- All transfections of plasmids, RNA extractions and consequent RT-PCR analysis were done as previously described (Revil *et al.*, 2007) as were treatments with RNAi (Garneau *et al.*, 2005). The target sequences for siK1 and siK2 were respectively GAGCGCAUAUUGAGUAUCA and UCUAGCAGGAGGAAUUAUU.

Transcription and splicing assays- In vitro transcription and splicing of minigene 45 and derivatives, as well as RT-PCR analysis of the products, were done as described previously (Nasim *et al.*, 2002). Templates for the RNA transcripts used in the immunoprecipitation were amplified by PCR (PfuTurbo, Stratagene) and gel purified (Qiagen). Transcription was done using T3 RNA polymerase (Promega) and [α-^{32}P]-CTP (Perkin-Elmer).

RNA chromatography- Coupling the RNA (IDT) to the beads and incubation of nuclear extracts in splicing conditions was done as previously described (Paradis *et al.*, 2007). After incubation, the beads were washed twice with eight volumes of KCl-free buffer D (60 mM HEPES, (pH 7.9), 0.2 mM EDTA, 0.5 mM phenylmethyl-sulfonyl fluoride, 0.5 mM dithiothreitol, and 20% glycerol) then eluted twice with four volumes of buffer D containing

100 mM KCl. These were pooled then precipitated by adding 1 volume of trichloracetic acid, incubating 20 min on ice and spinning 5 min at 10,000 g. The pellets were resuspended in NaOH 0.1 N. These steps, starting with washing with the previous eluting KCl concentration, were repeated for buffer D containing 250, 500 and 1000 mM KCl. The various eluates were loaded on a 10% polyacrylamide gel, stained with silver nitrate (Invitrogen) and the bands of interest were analyzed by mass spectroscopy.

RNA immunoprecipitation- Sepharose-protein A beads (GE) were incubated in buffer A (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 2.5 mM MgCl2) containing 0.5% Triton X-100 for one hour at 25°C, washed three times in the same buffer, then resuspended for a final volume of 50% beads. One µl of 12G4 anti-hnRNP K antibodies (kindly provided by G. Dreyfuss) was added per 50 µl of 50% slurry and incubated for one hour at 4°C. The beads were washed three times with cold buffer A. Equivalent quantities of transcripts were incubated in 12.5 µl of splicing mix (Nasim *et al.*, 2002) for 30 minutes at 4°C then washed five times with 1 ml of buffer A. Proteinase K and SDS was added to the beads and then incubated at 37°C for 15 minutes. Following phenol extraction and ethanol precipitation, the RNA was resuspended in formamide dye and loaded on a 6.5% denaturing acrylamide gel. The gels were then analyzed on a PhosphorImager and the results adjusted for relative amounts of radioactivity.

RESULTS

Mapping of enhancer and silencer elements upstream of the 5' splice site of Bcl-xS. Previous deletion mutagenesis using Bcl-x minigenes (Garneau *et al.*, 2005; Revil *et al.*, 2007)

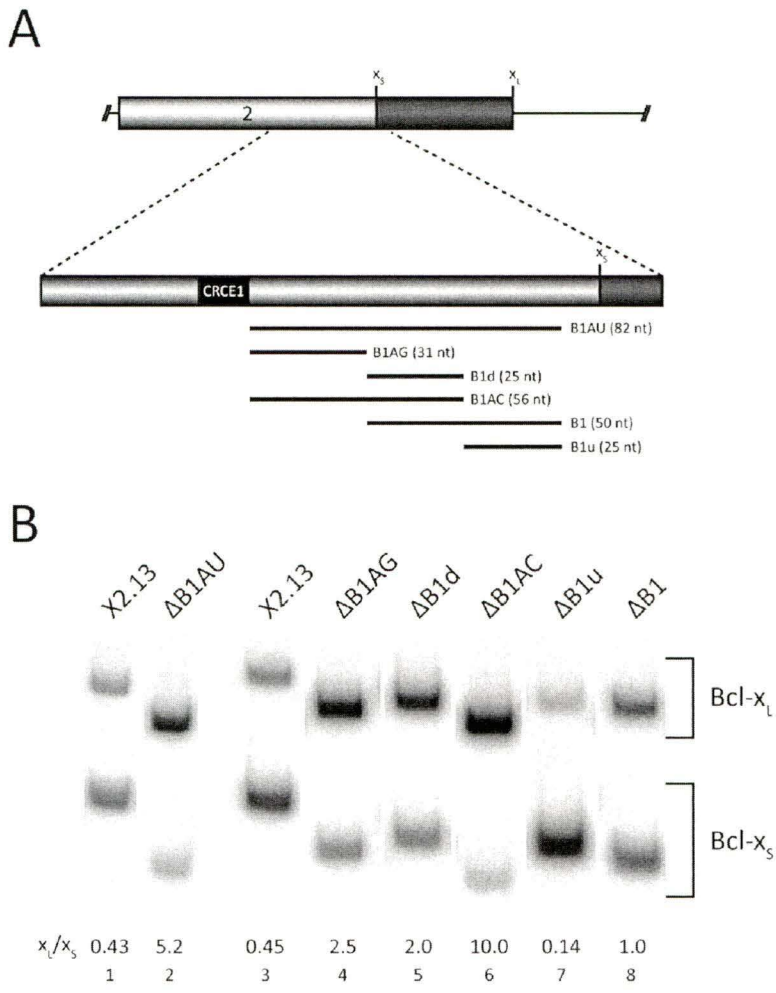

Fig. 2. Deletion mutagenesis of the B1 region leads to the discovery of three novel splicing regulating elements. *A.* The locations of the deletions are indicated by the bars and their sizes are shown in nucleotides (nt). *B.* Radio-labeled RT-PCR assays on total RNA extracted from HeLa cells transfected with the different deletion mutations. After separation on acrylamide gels, the bands were analyzed using a PhosphorImager. The corresponding Bcl-x_L and Bcl-x_S bands are shown, as well as the Bcl-x_L/Bcl-x_S ratio displayed above the lane numbers. The results presented are representative of many independent experiments.

transfected in HeLa cells identified a 82 nt region (B1AU; Fig. 2A), starting 10 nt upstream of the 5' splice site of Bcl-xS, that regulates Bcl-x splicing. When this region is deleted from the X2.13 minigene carrying the two 5' splice sites of Bcl-x, the Bcl-xL/Bcl-xS splicing ratio is increased by tenfold, as judged by RT-PCR analysis (Fig. 2B, compare lane 2 with lane 1). Thus, B1AU behaves as an enhancer for the Bcl-xS 5' splice site. Further dissection of B1AU was performed by dividing the element into three parts. Deletion of the upstream B1AG

portion (32 nt) also decreased the relative usage of the Bcl-xS site (Fig. 2B, compare lane 4 to lane 3). Removing the central portion of B1AU (B1d; 25 nt) had a similar effect (Fig. 2B, lane 5). The deletion of both B1d and B1AG to produce ΔB1AC increased splicing to the Bcl-xL 5′ splice site more than the impact of the two individual deletions (Fig. 2*B*, *lane 6*), indicating the presence of at least two functionally distinct enhancer elements. Furthermore, the impact of the B1AC deletion was even stronger than that of removing B1AU, suggesting that the downstream portion of B1AU (B1u; 25 nt) might possess silencer activity. Indeed, removing only B1u enhanced the use of the Bcl-xS splice site, consistent with the presence of a splicing silencer in this region (Fig. 2B, lane 7). Deleting both B1d and B1u (B1, Fig. 2B, lane 8) also decreased the effect relative to the deletion of B1d alone (lane 8). Thus, B1AU contains two antagonistic regions composed of at least three elements; B1AG and B1d, acting as enhancers and B1u behaving as a repressor of Bcl-xS usage. The activities of the enhancer (B1AC) and silencer (B1u) elements were similarly detected when the analysis was carried out in human 293 and PC-3 cells (data not shown).

Mutational analysis of B1AG, B1d and B1u. To identify nucleotides important for the activity of the different elements, a collection of 42 mutants was constructed, each one containing a dinucleotide mutation in B1AG, B1d or B1u (Fig. 3). Each mutant was transfected in HeLa and 293 cells and the x_L/x_S splicing ratio of plasmid-derived transcripts were determined by RT-PCR. In Figure 3A, this ratio is expressed relative to the ratio obtained with the wild-type X2.13 minigene construct. This analysis was performed independently at least three times (including once in triplicate). The experiment presented in Figure 3 represents one of such experiments and although the amplitude of the effect varied between independent experiments performed on different days, the trend was highly reproducible (not shown).

Because most of the mutations in B1AG reduced splicing to the Bcl-xS site (Fig. 3A, left panel), we conclude that B1AG is an enhancer. Disruptions anywhere in the contiguous stretch of the central 16 nt (AACUGCGGUACCGGCG) shifted splicing towards Bcl-xL. In silico analysis of these nucleotides using ESEfinder (Cartegni *et al.*, 2003; Smith *et al.*, 2006) identified putative ASF/SF2 binding sites (underlined in Fig. 3), and most of the mutations that reduce the strength of the sites also compromised usage of Bcl-xS. Intriguingly, mutating the nucleotides recently described as important for the binding of RBM25 (Zhou *et al.*, 2008) (boxed in the figure) had no effect.

A similar mutational analysis of B1d yielded a more nuanced conclusion (Fig. 3*A*, *middle panel*). The impact of mutating the first 14 nucleotides of B1d was consistent with the existence of a splicing enhancer targeting Bcl-x_S, and a contiguous stretch of six nucleotides (GACAUC) that may represent a binding site for ASF/SF2 (underlined) appeared important for this activity. In contrast, the mutations that had the most impact in the remaining downstream portion of B1d improved Bcl-x_S usage, a result that is more consistent with the presence of a silencer element. If a silencer element exists in B1d, it is not the dominant activity since deleting or transplanting B1d (see Fig. 4) indicates that its global activity is that of an enhancer.

Lastly, dinucleotide mutations in B1u generally increased the use of Bcl-x_S, consistent with this region being a silencer (Fig. 3*A*, *right panel*). The most important sequence was a stretch of eight nucleotides (AUAUCAGA) that represent putative binding sites for SRp40. However, mutations that did not affect the putative SRp40 binding site had a strong effect on Bcl-x

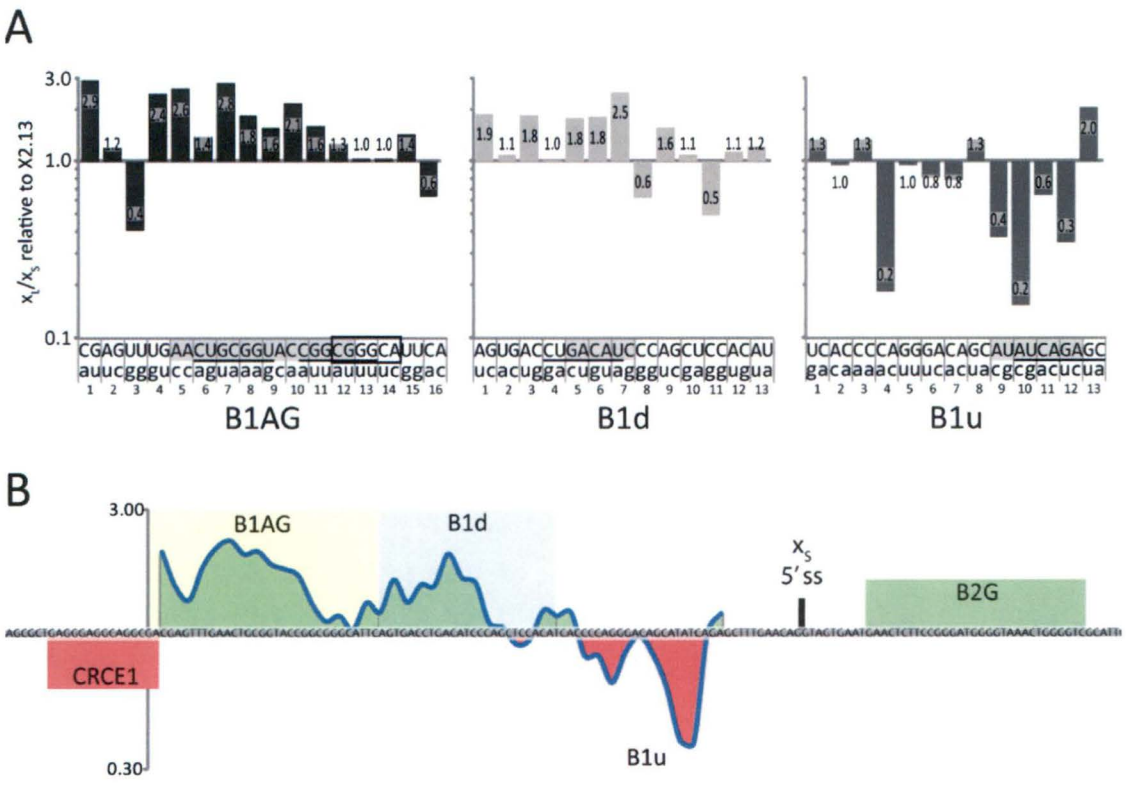

Fig. 3. Point mutations in the three elements affect bcl-x splicing. *A.* The mutations for every two nucleotides are represented in lower case beneath the wild-type sequence. Shown in the graph are Bcl-x_L/Bcl-x_S ratios for each mutation, relative to the Bcl-x_L/Bcl-x_S ratio of the minigene X2.13 which is represented by the horizontal line at 1.0. The nucleotides deemed important are highlighted in gray, while the putative binding sites for ASF/SF2 (B1AG and B1d) and SRp40 (B1u) are underlined. The RBM25 binding site recently discovered is boxed. *B.* Topological map representing the average of the effect of the mutational analysis. The value for each mutation was calculated as an average of it and both adjoining mutations. For example, the value for mutation B1d.7 is an average of the values in Fig. 3*A* for the mutations B1d.6, B1d.7 and B1d.8.

splicing (Fig. 3*A*, *right panel*, mutation 9). Moreover, knocking down SRp40 in a variety of cell lines did not significantly affect Bcl-x splicing (data not shown).

Globally, the results of the mutational analysis match very well the results of the deletions. Because splicing regulatory factors like SR and hnRNP proteins often have degenerate binding sites, not all mutations may alter in the same way the activity of a control element. Moreover, some mutations may create an element that imposes new splicing control. To facilitate the graphical representation of our results and to minimize the above caveats, we

assigned to each mutation a value that is an average of the impact of the mutation and the two adjoining mutations, thus more accurately displaying the contribution of existing elements (Fig. 3*B*). The most important feature of this map is a transition from enhancer to silencer activities occurring in B1d. Although B1d globally behaves as an enhancer due to its 5' portion, its 3' portion appears to represent the extremity of a silencer that extends into B1u.

The enhancer elements can function when placed in a different context. Mutations may modify alternative splicing by changing the secondary structure near a splice site or because they compromise the interaction with a trans-acting regulatory factor. To assess the intrinsic modulatory activity of the elements on alternative splicing, we inserted each one into a previously characterized reporter gene (Nasim *et al.*, 2002) containing the 5' splice sites of exon 7 and 7B of hnRNP A1, their surrounding sequences and the 3' splice site of the adenovirus major late exon L2 (Fig. 4*A*). The resulting minigenes were transcribed *in vitro* and the resulting pre-mRNAs were incubated in HeLa extracts for 2 hours. RT-PCR analysis was conducted to assess the splicing behavior of the inserted element. Placing B1AG upstream of the proximal 5' splice site improved its use (Fig. 4*B*, compare *lane 2* with *lane 1*). When the other enhancer element (B1d) was inserted at the same position, proximal 5' splice site usage was also increased (Fig. 4*B*, *lane 3*). Finally, the silencer element B1u had very little impact on splicing (*lane* 4). These results were confirmed by testing the various transcripts in several batches of nuclear extracts (not shown). Our results therefore suggest that the enhancer activities of B1AG and B1d are mediated by trans-acting factor(s). The activity of B1u does not appear transplantable (Fig. 4*B*, *lane 4*), suggesting that B1u may promote splicing repression by preventing the activity of the upstream enhancers.

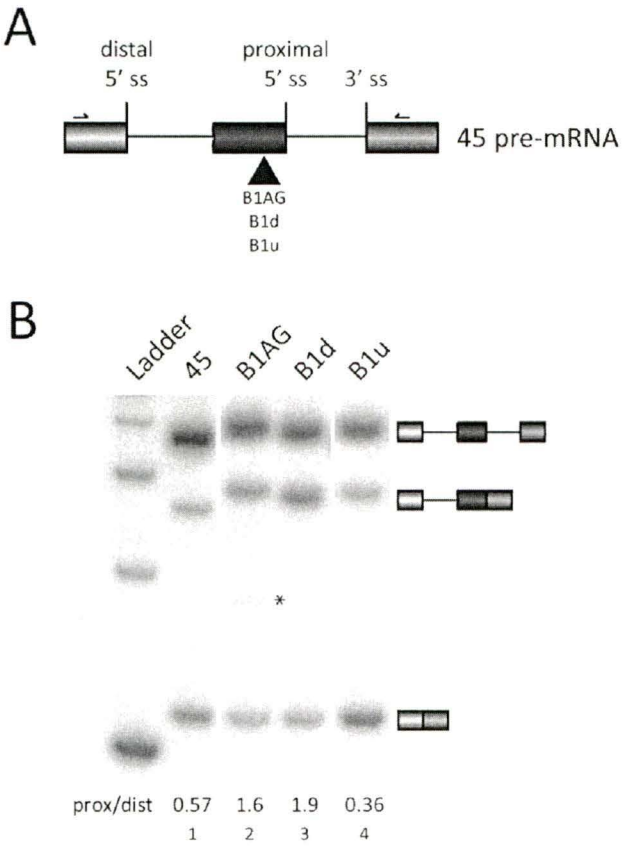

Fig. 4. The insertion of the enhancer elements in the minigene 45 replicates their activity. *A.* Each element was inserted upstream of the proximal 5' splice site of the minigene 45 as indicated. The primers used for subsequent RT-PCR analysis are shown. *B.* The transcripts derived from minigene 45 were incubated in HeLa nuclear extracts in *in vitro* splicing conditions. RT-PCR analysis was done on total RNA. The splicing products are indicated as well as the proximal/distal ratios. A new product presumably produced by a cryptic splice site is indicated with an asterisk.

HnRNP K binds to the B1 element. To isolate factors that mediate the activity of these control elements, we carried out affinity chromatography with RNA portions covalently linked to agarose beads using HeLa nuclear extracts. The bound material was eluted with increasing amounts of KCl. The content was fractionated on acrylamide gels and revealed by silver staining; bands were cut and proteins were analyzed by nanoliquid chromatography coupled online with tandem mass spectrometry (data not shown). The strongest hit obtained came

with B1d for which a 60 kDa protein that eluted at 250 mM KCl was identified as hnRNP K. Binding of hnRNP K may be explained by the presence of UCCCAG and UCCACAU, which are sequences very similar to the high-affinity RNA binding site for hnRNP K (UC_{3-4}(AU)/(UA)) identified through SELEX (Thisted *et al.*, 2001). Our previous analysis in Figure 3*A* showed that mutating the two central cytidines of either sequences stimulated splicing to the 5' splice site of Bcl-x_S, consistent with the view that hnRNP K might repress the production of Bcl-x_S.

To further analyze the interaction of hnRNP K with the Bcl-x pre-mRNA we carried out an RNA immunoprecipitation assay using our model Bcl-x pre-mRNA (X2.13) and versions carrying various deletions. Radio-labeled transcripts were incubated in HeLa nuclear extracts under *in vitro* splicing conditions. HnRNP K antibody (a kind gift from G. Dreyfuss) conjugated to protein A Sepharose was then added. After several washes, the labeled RNA was recovered and quantitated on gel. This assay revealed that the binding of hnRNP K to X2.13 strongly decreased when B1, B1d or B1u were deleted (Fig. 5). This interaction of hnRNP K with B1u may be explained by the presence of a contiguous stretch of four C, a known recognition site for hnRNP K (Bomsztyk *et al.*, 2004). Consistent with this possibility, the mutation B1u.4, which changes the last cytidine of that stretch and severely compromises the activity of the silencer, diminished the recovery of the RNA by anti-hnRNP K immunoprecipitation. Mutating the abutting CC into AA did not compromise recovery with the anti-hnRNP K antibody nor did it affect Bcl-x splicing (Fig. 3*A*). Notably, mutation B1d.7, which has an effect opposite than that of deleting B1u (Fig. 2*B* and Fig. 3*A*) slightly but significantly increased recovery of the RNA by anti-hnRNP K immuno-precipitation. This

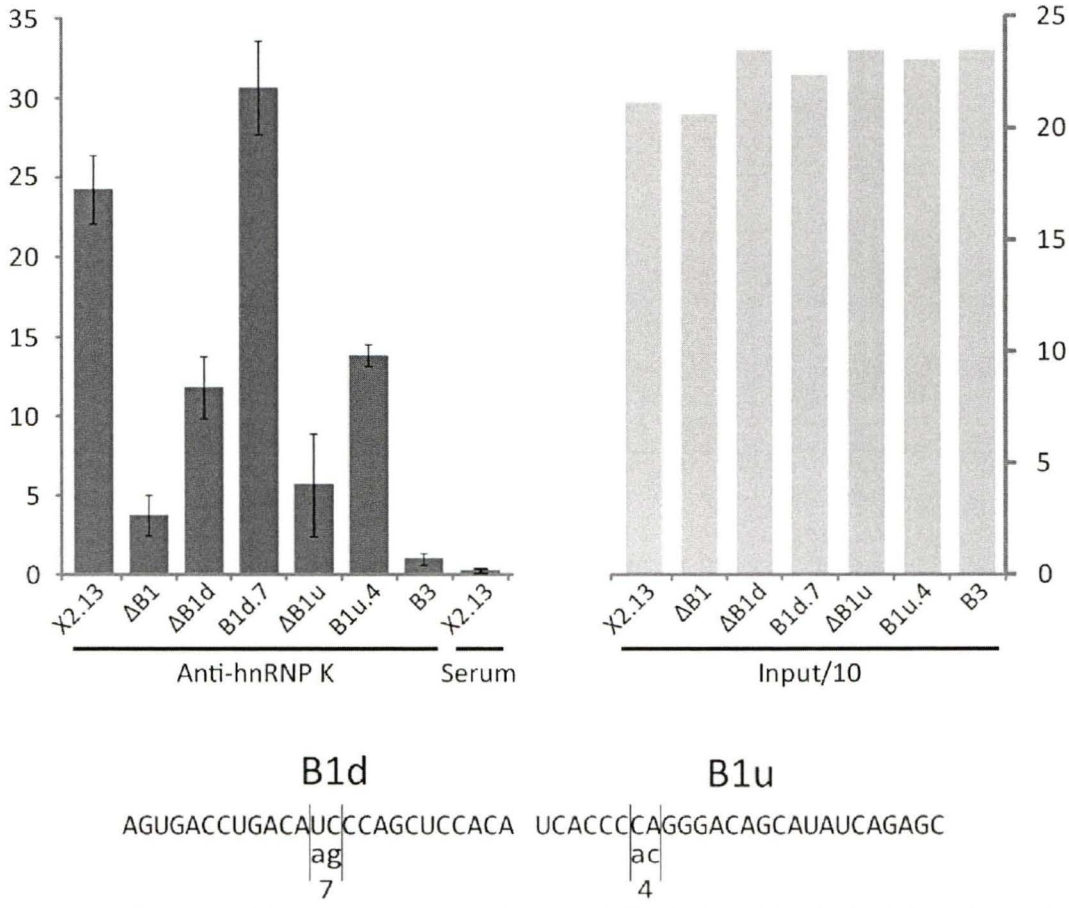

Fig. 5. RNA co-immunoprecipitation of a portion of the bcl-x pre-mRNA using hnRNP K antibodies is dependent on the B1 region. The transcripts were incubated in HeLa nuclear extracts in splicing conditions then on beads coupled with hnRNP K antibodies (Anti-hnRNP K) or pre-immune serum (Serum). After five washes, the RNA was precipitated, migrated on an acrylamide gel and quantified on a PhosphorImager. On the left panel is a graph of the values of radioactivity measured. The right panel shows the relative levels of a tenth of the input RNA. The sequence of the point mutations that were tested is indicated in the bottom panel.

mutation may enhance hnRNP K binding directly or indirectly by disrupting a binding site for an enhancer protein that competes with hnRNP K for binding in the region where the transition between enhancer and silencer activities occurs. We could not confirm direct binding of hnRNP K because our recombinant hnRNP K protein produced in bacteria lacked any type of binding activity.

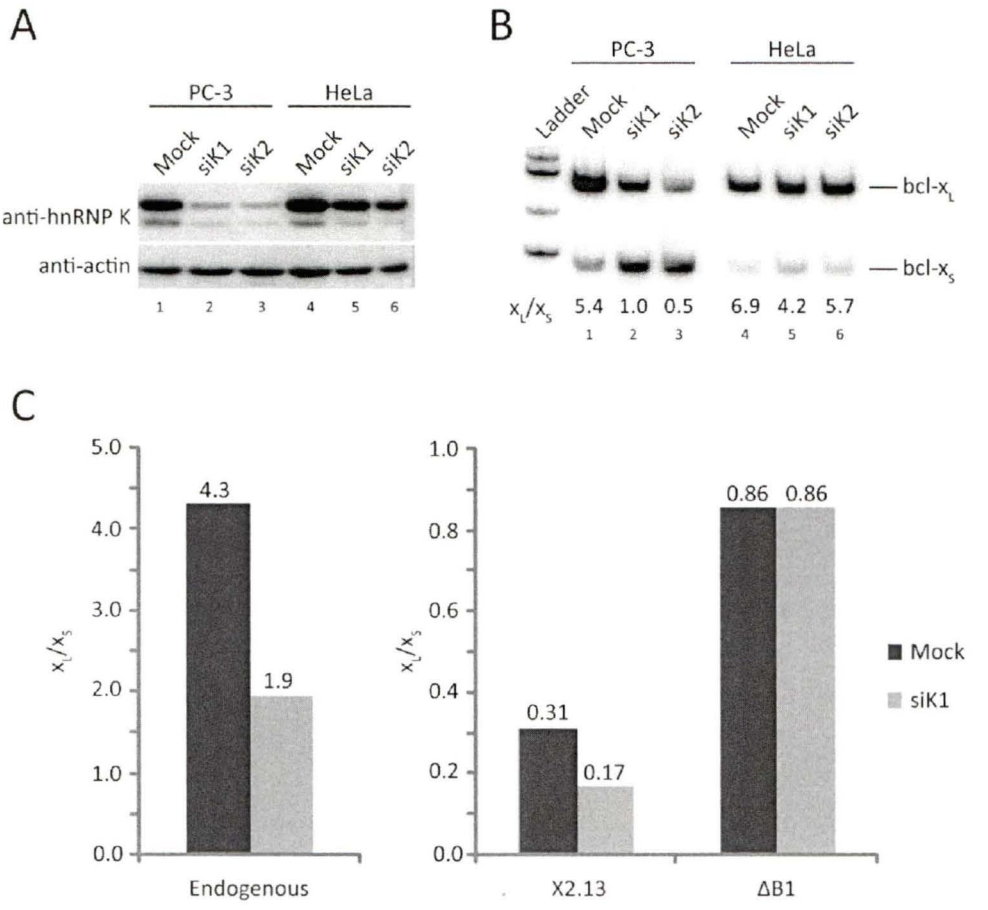

Fig. 6. The knockdown of hnRNP K affects Bcl-x splicing in a B1-dependent way. *A.* Knockdown of hnRNP K was done in PC-3 and HeLa cells using two different siRNAs (siK1 and siK2) or mock-transfected (Mock). Actin was used as a loading control. Proteins loaded on an acrylamide gel were revealed using antibodies against hnRNP K and actin. *B.* RT-PCR analysis of endogenous Bcl-x mRNAs using the RNAi-treated PC-3 and HeLa cells. The Bcl-x_L/Bcl-x_S ratios are indicated above the lane numbers. *C.* siK1 RNAi-treated PC-3 cells were mock-transfected or transfected with the wild-type minigene or its ΔB1 version. Twenty-four hours later, total RNA was extracted and analyzed using radiolabeled RT-PCR with primers for endogenous Bcl-x (for mock-transfected cells) or primers specific for the minigenes. The graphs represent the Bcl-x_L/Bcl-x_S ratios for each sample.

Knockdown of hnRNP K affects Bcl-x splicing in a B1-dependent manner. In contrast to other abundant hnRNP proteins like hnRNP A1 and hnRNP I/PTB, there is little evidence supporting a direct role for hnRNP K in splicing control (Expert-Bezancon *et al.*, 2002; Griffith *et al.*, 2006; Ule *et al.*, 2006). The related Nova proteins contain KH-domains found in prototypical hnRNP K and they regulate brain-specific alternative splicing events (Licatalosi *et al.*, 2008; Ule et Darnell, 2007; Ule *et al.*, 2006). To clarify the contribution of hnRNP K to Bcl-x splicing,

we knocked down hnRNP K expression by RNA interference using two different non-overlapping siRNAs (siK1 and siK2) in PC-3 and HeLa cells. The knockdown was successful in PC-3 cells but was less efficient in HeLa cells (Fig. 6*A*). Assessing the impact of the knockdowns on the endogenous Bcl-x splicing profiles in PC-3 cells revealed an increase in the use of the Bcl-x_S 5′ splice site (Fig. 6*B*, *lanes* 1-3), consistent with the notion that hnRNP K represses the splicing of Bcl-x_S. The effect was less dramatic in HeLa cells (*lanes* 4-6) as expected from a more limited depletion. We observed increased Bcl-x_S isoform formation upon siRNA treatment in several other cell lines (data not shown). To confirm that the modulation of splicing by hnRNP K requires the B1 element, we looked at the impact of the hnRNP K depletion on the splicing of transcripts produced from minigenes X2.13 containing or lacking the B1 element (Fig. 6*C*). Only the wild-type X2.13 minigene experienced a decrease in the Bcl-x_L/Bcl-x_S ratio when hnRNP K was depleted in PC-3 cells. Thus, our results indicate that the activity of hnRNP K is mediated through B1, most likely through the C-rich elements important for hnRNP K binding in B1d and B1u.

DISCUSSION

Given the functional importance of the major Bcl-x splice isoforms in apoptosis and their antagonistic roles, it is not surprising that the splicing decisions that occur on the Bcl-x pre-mRNA are regulated by a variety of elements and factors (Boon-Unge *et al.*, 2007; Chalfant *et al.*, 2002; Cloutier *et al.*, 2008; Garneau *et al.*, 2005; Li *et al.*, 2004b; Massiello *et al.*, 2006; Massiello *et al.*, 2004; Paronetto *et al.*, 2007; Revil *et al.*, 2007; Zhou *et al.*, 2008). Many of these elements may help link splicing regulation with specific pathways that monitor the

integrity of various cellular components and compartments as well as the availability of the nutrients and growth conditions.

In the present report, we document the activity of an 82 nt region (B1AU) located immediately upstream of the 5' splice site of Bcl-x_S. The 5' portion of this region displays enhancer activity since deleting or mutating specific nucleotides decreases the use of the Bcl-x_S site. Because portions of this region can function in a heterologous context, the activity of the enhancer likely requires trans-acting factor(s). The region contains three putative high-affinity binding sites for ASF/SF2. However, a role for ASF/SF2 in the activity of these elements is unlikely because overexpressing recombinant ASF/SF2 in a variety of cell lines increases the production of Bcl-x_L rather than that of Bcl-xS (Cloutier *et al.*, 2008; Li *et al.*, 2005; Massiello et Chalfant, 2006; Paronetto *et al.*, 2007). Recently, RBM25 was identified as binding to a six nucleotides element (CGGGCA) in B1AG (Zhou *et al.*, 2008). Although the activity of RBM25 in HeLa cells is consistent with the enhancer activity of B1AG, our mutational analysis did not reveal a role for these sequences both in 293 and HeLa cells (Fig. 3 and data not shown). Perhaps mutating only two nucleotides at a time was not enough to destroy the activity of this sequence element. The SR protein SC35 has also been implicated as mediating the increase in Bcl-xS upon treatment of various cell lines with doxycyclin or cyclophosphamide (Merdzhanova *et al.*, 2008). However, these drugs and the knockdown of SC35 did not affect Bcl-x splicing in HeLa and PC-3 cells (data not shown). Further work will be required to identify the factors that are responsible for the enhancing activity of B1AG and B1d.

We also mapped a silencer occupying the 3' half of the BIAU element. RNA affinity chromatography using a sub-region identified hnRNP K, a protein that was previously reported to stimulate inclusion of the β-tropomyosin exon 6A (Expert-Bezancon *et al.*, 2002) but acting as a repressor in the glucose-6-phosphate dehydro-genase pre-mRNA (Griffith *et al.*, 2006). hnRNP K can also bind to the Nova1 pre-mRNA and can decrease the inclusion of alternative exon 4 (Ule *et al.*, 2006). Recently, it was shown that hnRNP K can play a prominent role in alternative splicing control since nearly half of 56 alternative splicing events in apoptotic genes were affected upon hnRNP K depletion, either enhancing or suppressing exon inclusion (Venables *et al.*, 2008b). This broad role in splicing regulation may come from its ability to interact with several splicing-related proteins, including other hnRNP proteins (Bomsztyk *et al.*, 2004; Mikula *et al.*, 2006). As with other hnRNP proteins, hnRNP K has also been implicated in other steps of gene expression including transcription and translation (Bomsztyk *et al.*, 2004). For example, hnRNP K activates transcription of c-myc and serves as a cofactor for p53-dependent transcriptional activation following DNA damage (Lee *et al.*, 1996; Moumen *et al.*, 2005), but can also repress transcription of thymidine kinase and CD43 (Da Silva *et al.*, 2002; Hsieh *et al.*, 1998). hnRNP K also represses translation of p21 and c-src during neuronal and erythroid differentiation, respectively (Naarmann *et al.*, 2008; Yano *et al.*, 2005).

In the case of Bcl-x, hnRNP K binds to portions of B1 containing C-rich elements that are similar to the high-affinity sites identified by SELEX (Thisted *et al.*, 2001). These sites are located in the silencer portion of B1AU and, consistent with a role for hnRNP K in repressing the use of Bcl-xS, depleting hnRNP K by RNAi provokes a B1-dependent increase in the production of Bcl-xS. Our mutational analysis indicates that the silencer element occupies

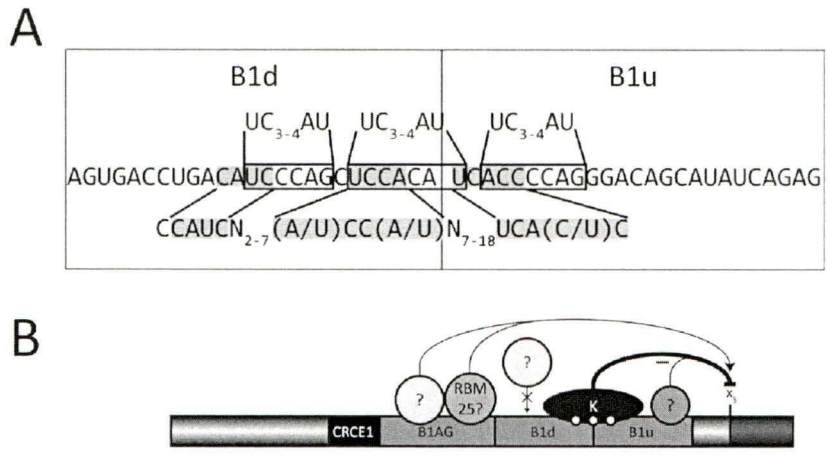

Fig. 7. *A*. Putative binding sites for hnRNP K in the B1d and B1u elements. The three sites resembling the high-affinity binding sites are boxed and compared to those identified by SELEX (49), shown above the RNA sequence for the B1 element. Below is the binding site defined using yeast three-hybrid screens (67,68), compared to the shaded binding site found on the Bcl-x pre-mRNA. *B*. hnRNP K binding to B1 may compete with the binding of an unknown enhancer protein, thus inhibiting splicing to the Bcl-x_S site. Deletion of the enhancer element B1AG augments the use of the Bcl-x_L splice site. When the other enhancer element, B1d, is removed, the inhibition by hnRNP K on the x_S 5′ splice site would be slightly diminished, perhaps by the stronger binding of a protein to B1AG or reduced binding affinity of hnRNP K to the pre-mRNA. Removal of the silencer B1u would abrogate hnRNP K binding leading to a strong increase in the production of the Bcl-x_S isoform, as does a knockdown of hnRNP K by RNA interference.

the 3′ end of B1d and all of B1u. Individually mutating the two central cytidines of the two putative high-affinity binding sites for hnRNP K in B1d abrogated the repression, consistent with a direct role for hnRNP K in the activity of this portion of B1d. Thus, B1d likely represents a pivotal region because it is bound by both positive and negative control factors. Another putative binding site for hnRNP K is present in B1u (ACCCCA; Fig. 7A). Mutating the last two nucleotides strongly diminished the repression of Bcl-xS, as well as decreased binding of hnRNP K.

Another high-affinity binding site that was previously identified using yeast three-hybrid screens is CCAUCN2-7(A/U)CC(A/U)N7-18UCA(C/U)C (Klimek-Tomczak *et al.*, 2004; Paziewska *et al.*, 2004). B1d and B1u contain ACAUCCCAGCUCCA-CA|UCACCCCA (the vertical

line indicates the division between both elements). Thus, three putative hnRNP K binding sites exist within a stretch of 25 nucleotides and cooperative interactions may stabilize the binding of hnRNP K and antagonize the binding of positive factors in the central portion of B1d. The fact that B1u displays little activity when transplanted in a different pre-mRNA is consistent with a model in which the role of the silencer would be to antagonize the binding or activity of flanking activators. The silencer element is likely to be more complex than the binding of hnRNP K alone since the 3' end of B1u is the most active silencer region and yet it lacks putative hnRNP K binding sites. A simple working model for the antagonizing activities associated with B1AU is presented in Figure 7B. The enhancer activity would be mediated by factor(s) interacting with the 5' half, perhaps RBM25. The central portion contains sequences bound by hnRNP K but this region may overlap with binding sites for the enhancing factors. Two putative hnRNP K binding sites already exist in B1d and a third one is likely present in B1u. As indicated in the introductory section, overlapping binding sites for factors with different activities are often used to control splicing decisions in other pre-mRNAs (Expert-Bezancon *et al.*, 2004; Paradis *et al.*, 2007; Swanson et Stoltzfus, 1998; Zahler *et al.*, 2004).

HnRNP K can interact with Sam68 in vitro and in vivo (Gorla *et al.*, 2006; Yang *et al.*, 2002), a protein known to regulate Bcl-x splicing (Paronetto *et al.*, 2007). However, while we have shown that hnRNP K represses the production of Bcl-xS, Sam68 stimulates it. Although it is unclear where Sam68 binds, hnRNP K may neutralize Sam68 by interacting with it when these proteins are in close proximity. However, performing a knockdown of Sam68 had no effect in our conditions and cell lines (data not shown).

The ability of hnRNP K to repress the production of the pro-apoptotic Bcl-x_S isoform would confer to hnRNP K an anti-apoptotic function that may help cancer cells escape death signals. As shown recently, the knockdown of hnRNP K can influence the alternative splicing of several apoptotic genes (Venables *et al.*, 2008b), including favoring the production of the pro-apoptotic splice form of MCL1 (B.C, unpublished results). MCL1 is another BCL2 family member and its pro-apoptotic variant is consistently underproduced in breast cancer tissues (Venables *et al.*, 2008a). An anti-cell death function for hnRNP K is also suggested by the increase in the nuclear concentration of hnRNP K that occurs in proliferating cells as well as in tumors (Ostrowski et Bomsztyk, 2003), and the higher level of hnRNP K observed in a variety of cancers compared to the corresponding healthy tissues (Carpenter *et al.*, 2006; Dejgaard *et al.*, 1994; Hatakeyama *et al.*, 2006; Li *et al.*, 2004a; Mandal *et al.*, 2001; Pino *et al.*, 2003; Roychoudhury et Chaudhuri, 2007). Overexpression of hnRNP K also led to the transformation of Rat1A cells (Lynch *et al.*, 2005). Since DNA damage transiently increases the production of hnRNP K in many human cancer cell lines (Moumen *et al.*, 2005), this response may be used to further repress the production of apoptotic isoforms, hence creating conditions to implement DNA repair without triggering apoptosis. Through its role in splicing, hnRNP K may therefore help coordinate the DNA damage response with apoptotic regulation.

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FOOTNOTES

*We thank Gideon Dreyfuss for the hnRNP K antibodies, Shu-Ching Huang for the RBM25 antibodies and Catherine Desrosiers for Taq polymerase. We also thank Uli Froehlich and the Laboratory of Functional Genomics of the Université de Sherbrooke for providing reagents. We are grateful to Laetitia Michelle and Lulzim Shkreta for discussions.

The abbreviations used are: hnRNP, heterogeneous ribonucleoprotein particles; RT-PCR, reverse transcription-PCR; siRNA, small interfering RNA; siK, siRNA targeting hnRNP K; snRNP, small nuclear ribonucleoprotein.

Discussion

L'épissage alternatif du pré-ARNm de *bcl-x* permet de générer deux isoformes majoritaires, par l'utilisation de deux sites d'épissage 5' dans l'exon 2. Ces deux isoformes possèdent des activités antagonistes : l'utilisation du site d'épissage 5' proximal produit l'isoforme Bcl-x_L encodant une protéine qui augmente la survie de la cellule, tandis que l'utilisation du site d'épissage 5' distal forme Bcl-x_S qui favorise la mort cellulaire.

Dans le laboratoire, nous étudions la régulation de l'épissage alternatif de *bcl-x* dû à cette dualité intéressante de l'activité de ces isoformes. Dans ce but, nous avons créé un mini-gène contenant une partie de l'exon 2 de *bcl-x*, ses deux sites d'épissage 5', 1,2 kb de l'intron après le site d'épissage de Bcl-x_S ainsi que le site 3' de l'exon 3 (Cloutier *et al.*, 2008; Garneau *et al.*, 2005; Revil *et al.*, 2008; Revil *et al.*, 2007). Auparavant, la comparaison des séquences des gènes de *hnRNP A1* chez la souris et l'homme avait permis d'identifier plusieurs séquences conservées dans l'intron qui ont ensuite été démontrées comme ayant une activité dans l'épissage alternatif (Blanchette et Chabot, 1997; Chabot *et al.*, 1997; Simard et Chabot, 2000). La même étude pour le gène de *bcl-x* entre la souris et l'homme (T. Revil, D. Garneau et B. Chabot, données non publiées) n'a pas permis d'identifier d'éléments potentiels car son intron est très conservé. Ceci est possiblement dû à l'existence de l'isoforme protéique Bcl-x_β qui est encodé par une partie de l'intron (Ban *et al.*, 1998; Bianchini *et al.*, 2006; Shiraiwa *et al.*, 1996). Nous avons donc effectué des délétions dans le mini-gène afin de tenter de cibler des régions importantes pour la régulation de l'épissage de *bcl-x*. Une première série de délétions ciblait la région intronique de *bcl-x*. Aucune de ces

délétions ne semblaient avoir d'effet dans l'épissage *in vitro* ou *in vivo* (T. Revil, D. Garneau et B. Chabot, données non publiées). Cette absence d'effet serait peut-être due aux types cellulaires que nous avons utilisés pour notre étude, soit les cellules HeLa, provenant de cancer du col de l'utérus, et les EcR-293, des cellules embryonnaires hépatiques transformées. Dans les cellules de leucémie K562 ou de gliomes U251, il a été démontré que l'intron possède une activité de réponse aux cytokines IL-6 et GM-CSF ainsi qu'au TPA, un activateur de kinases (Li *et al.*, 2004b). Il est possible que ces cellules possèdent un facteur qui est absent dans les types cellulaires que nous avons vérifiés, ou qu'au contraire il leur manque un autre facteur les rendant plus sensibles à cette régulation.

Par la suite, des délétions effectuées dans l'exon 2 du mini-gène ont permis de cibler plusieurs régions importantes qui régulent l'épissage alternatif de *bcl-x*. L'élément B2G est lié par les protéines hnRNPs F et H qui augmentent l'utilisation du site d'épissage x_S (Garneau *et al.*, 2005). L'élément B3 contient deux sites de liaison pour SRp30c qui favorisent la formation de Bcl-x_L, ainsi que deux sites d'épissage 5' cryptiques qui l'inhibent par la liaison du snRNP U1 (Cloutier *et al.*, 2008). Outre ces deux régions, plusieurs autres éléments, facteurs, et voies de signalisation ont été identifiés (Voir Introduction; Boon-Unge *et al.*, 2007; Li *et al.*, 2004b; Massiello *et al.*, 2006; Massiello *et al.*, 2004; Paronetto *et al.*, 2007; Zhou *et al.*, 2008). Au cours de ces années, j'ai participé à la découverte de deux autres régions régulant l'épissage alternatif de *bcl-x* (Revil *et al.*, 2008; Revil *et al.*, 2007).

Le contrôle de l'épissage alternatif par la protéine kinase C (PKC)

Dans le premier chapitre, nous avons démontré l'implication de la protéine kinase C sur l'épissage alternatif de *bcl-x*, dépendant de la présence de la région SB1. Durant notre

analyse des mécanismes régulant l'épissage alternatif de *bcl-x*, nous voulions un modèle permettant d'observer un plus grand ratio de l'isoforme x_S par rapport à x_L (x_S/x_L). Puisque l'apoptose induite par la staurosporine dans les cellules de foie Chang entraîne la baisse des protéines Bcl-x_L (Giuliano *et al.*, 2004), nous avons vérifié si cet effet était aussi visible au niveau de l'ARNm. Dans nos essais, sur les sept lignées cellulaires vérifiées, seule la lignée non-cancéreuse EcR-293 répondait à un traitement à la staurosporine par la production de l'isoforme de l'ARNm du pro-apoptotique Bcl-x_S et une baisse de l'isoforme de Bcl-x_L (Figure 2; Revil *et al.*, 2007). Ceci n'est pas dû à l'imperméabilité des autres cellules à cet agent puisqu'il était possible d'observer de la mort cellulaire chez toutes les lignées (Figure 15). L'effet de la staurosporine sur l'épissage de *bcl-x* dans les EcR-293 augmente avec le temps de traitement ainsi que la concentration utilisée. La modification du ratio n'est pas due à une stabilité différente des deux isoformes car l'inhibition de la transcription par un prétraitement avec le 5,6-dichloro-1-ß-D-ribobenzimidazole (DRB), bloquant ainsi la formation de nouveaux ARNm, empêche aussi l'effet de la staurosporine. Si la stabilité des ARNm était en cause, l'ajout de staurosporine aurait quand même un effet sur les transcrits déjà présents. De plus, la production de nouvelles protéines n'est pas nécessaire car l'inhibition de la traduction protéique par le cycloheximide (CHX) n'avait aucun effet sur l'augmentation de l'isoforme Bcl-x_S par la staurosporine. Ceci suggère que l'effet de la staurosporine passerait par des modifications post-traductionnelles d'un facteur déjà présent. Le CHX seul a de plus déjà un effet de base, augmentant l'utilisation du site

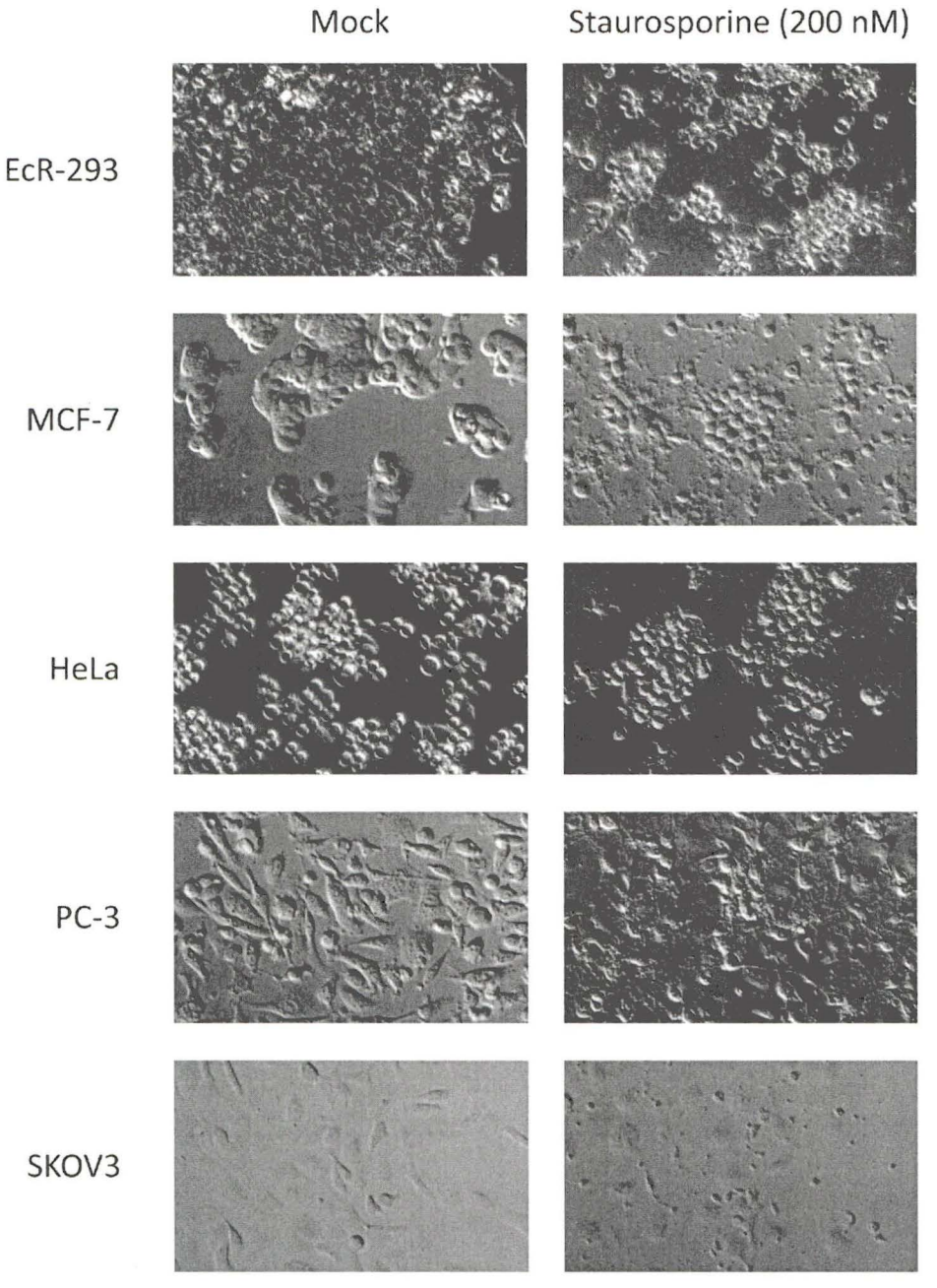

Figure 15 – La staurosporine induit l'apoptose. Photographies de cellules telles qu'observées par microscopie à contraste de phase. Le traitement des différentes lignées cellulaires avec 200 nM de staurosporine pendant 24 heures induisait l'apoptose telle que démontrée par la formation de corps apoptotiques.

d'épissage x_S. Ceci pourrait être dû à l'apoptose causée par cet agent dans certaines lignées cellulaires (Baskic *et al.*, 2006; Jiang *et al.*, 2008). Le cycloheximide peut aussi activer les protéines kinases activées par un mitogène (MAPK) ou stress (SAPK) (Zinck *et al.*, 1995). Ceci

pourrait interférer d'une manière indéterminée sur l'épissage alternatif de *bcl-x*. Cependant, même à des concentrations inhibant la synthèse protéique, l'ajout additionnel de staurosporine augmente encore plus la production de l'isoforme Bcl-x_S, indiquant que les modifications post-traductionnelles de protéines existantes pourraient entrer en jeu.

La staurosporine induisant l'apoptose, ceci pourrait activer les caspases qui cliveraient des facteurs d'épissage (Fischer *et al.*, 2003) et potentiellement modifier l'épissage alternatif. Nous avons donc prétraité avec un inhibiteur général de caspases, le z-VAD-fmk, et ceci permet toujours l'effet de la staurosporine (Figure 3; Revil *et al.*, 2007). De plus, la concentration (50 nM) de staurosporine utilisée ne semble même pas suffisante pour activer le clivage de la PARP, une étape de l'apoptose (Lazebnik *et al.*, 1994), comparativement à la vinorelbine. Puisque la staurosporine agit en inhibant plusieurs kinases avec une très haute affinité mais très peu de spécificité (Karaman *et al.*, 2008), nous avons voulu démontrer quelle kinase affectée était responsable de la modification du ratio x_S/x_L (Figure 3; Revil *et al.*, 2007). La staurosporine inhibe très fortement la PKC (Tamaoki *et al.*, 1986), ainsi nous avons vérifié et confirmé l'implication de cette kinase en utilisant deux inhibiteurs plus spécifiques, soit le Gö6976 et la calphostine C (Tamaoki et Nakano, 1990). L'effet du Gö6976 n'est encore visible que dans les EcR-293, comparativement aux trois lignées cancéreuses vérifiées.

Nous avons ensuite démontré que la staurosporine pouvait induire une modification de l'épissage alternatif du pré-ARNm *Axl*, et que ceci n'était encore une fois visible que dans les EcR-293 et dépendant de la PKC (Figure 4; Revil *et al.*, 2007). *Axl* est un récepteur tyrosine kinase jouant un rôle dans la prolifération cellulaire, la croissance et la survie cellulaire et sa

surexpression a été identifiée dans plusieurs types de cancer (Green *et al.*, 2006; van Ginkel *et al.*, 2004). Cependant, le rôle de l'isoforme court, qui est favorisé lors du traitement à la staurosporine, n'a pas encore été découvert. Puisque la production des isoformes différents d'*E1a* n'est pas affectée par l'ajout de staurosporine, l'inhibition de la PKC n'a donc pas un effet général sur l'épissage alternatif.

Afin de cibler la région du pré-ARNm de *bcl-x* responsable de l'effet de la staurosporine, nous avons utilisé un mini-gène contenant l'exon 2 au complet, soit X2. Malgré la présence d'un peu plus de Bcl-x_S que les transcrits endogène, l'épissage alternatif des transcrits provenant de X2 est néanmoins sensible à l'ajout de la staurosporine (Figure 5; Revil *et al.*, 2007). Lorsque nous enlevons la région SB1, soit une grande portion en 5' de l'exon 2, on forme le mini-gène X2.13 qui ne répond plus à la staurosporine. Cet effet est d'autant plus visible en utilisant des mini-gènes contenant des mutations augmentant la production de l'isoforme Bcl-x_L, soit X2-W et X2.13-W. Une autre observation intéressante est que la région SB1 inhibe la production de l'isoforme Bcl-x_S, si on compare X2 et X2.13 sans staurosporine, dans les cellules EcR-293 mais aussi dans les cellules HeLa. Cependant l'ajout de la staurosporine ne lève cette inhibition que dans les EcR-293. Ceci suggère la présence d'un facteur qui lie SB1 dans ces deux lignées cellulaires, quoique celui-ci ne réponde à l'inhibition de la PKC que dans les EcR-293. Il se pourrait que les cellules HeLa (et autres cellules cancéreuses vérifiées) ne possèdent pas une protéine nécessaire à l'inactivation du facteur liant SB1. Il est aussi possible que la voie de signalisation de PKC soit affectée dans les lignées cancéreuses. La suractivation des isozymes PKCα et PKCβ a été liée à la progression des cancers (Koivunen *et al.*, 2006) et l'expression des multiples isozymes de PKC est différente dans ceux-ci (Aaltonen *et al.*, 2006; Langzam *et al.*, 2001; Varga *et al.*, 2004). Ceci suggère

ainsi une réponse différente à l'inhibition de PKC par la staurosporine dans les lignées cellulaires cancéreuses. Il se peut cependant que ce soit la voie de signalisation de PKC de la lignée EcR-293 qui soit très sensible à la staurosporine. En effet, les cellules 293 ont été créées par transformation avec de l'ADN provenant de l'adénovirus 5. Elles expriment donc, entre autres, E1a qui peut promouvoir une relocalisation des PKC aux membranes (Shiroki *et al.*, 1992). Il sera intéressant de poursuivre l'étude de la voie de signalisation PKC-dépendante qui influence l'épissage alternatif de *bcl-x*. Une étudiante actuelle, Laetitia Michelle, poursuit cet aspect.

L'élément SB1 étant très grand, d'une longueur de 361 nt, ceci rend difficile l'identification d'une séquence et d'un facteur important pour l'épissage de *bcl-x*. Nous avons donc divisé celui-ci en trois morceaux se chevauchant afin de cibler plus précisément la région nécessaire pour l'activité de la staurosporine. Nous les avons insérés dans une unité d'épissage modèle Dup51, constituée de régions dupliqués du gène de la *β-globine* humaine, ainsi que dans une unité d'épissage dérivée du gène *E1a*. Étonnement, la staurosporine a un effet sur le mini-gène Dup51 contenant la portion C mais sur le mini-gène E1a contenant la portion B (Figure 6; Revil *et al.*, 2007). Comme ces deux portions se chevauchent, il est possible que l'élément de réponse à la staurosporine soit dans la région commune. De plus, puisque chaque unité d'épissage possède déjà son bagage de régulation de l'épissage alternatif, ceci pourrait interférer avec les différentes portions contenant aussi l'élément (i.e. le mini-gène Dup51+B ou E1a+C).

La comparaison des séquences B et C de la région SB1 avec l'exon alternatif de *Axl* régulé par la staurosporine a permis d'identifier des régions riches en CU communes aux deux. Ces

éléments peuvent être liés par hnRNP L. Cependant, une déplétion de cette protéine n'a pas eu d'effet lors de nos essais (T. Revil, J. Toutant et B. Chabot, données non publiées). Afin de simplifier l'analyse de la grande région SB1, j'ai démarré une étude par mutations ponctuelles qui est toujours en cours. Les portions B et C ont été mutées dix nucléotides à la fois dans le mini-gène X2 par Alexandre Cloutier et moi-même. Nous avons identifié ainsi cinq éléments potentiellement intéressants qui, lorsque mutés, annulent l'inhibition du site x_5 par la région SB1 X2 et/ou annulent l'effet de l'ajout de la staurosporine. Une analyse plus poussée qui sera effectuée par Alexandre Cloutier et Lulzim Shkreta permettra de cibler les nucléotides importants de ces éléments. De plus, j'ai aussi créé un mini-gène d'*Axl* contenant deux exons constitutifs et l'exon alternatif central. Il serait intéressant de vérifier si les transcrits provenant de celui-ci répondent à l'ajout de la staurosporine comme nous l'avons démontré pour les transcrits endogènes. Si c'est le cas, l'étude de mutations par délétion pour cibler la région importante pour cette régulation pourrait indiquer si l'élément nécessaire est le même que celui dans SB1.

Certains résultats obtenus dans le laboratoire suggèrent que la protéine impliquée dans la modulation de l'épissage alternatif de *bcl-x* par la voie de signalisation de PKC ne soit présente qu'en quantité limitée. L'inhibition de la traduction par le cycloheximide, l'émétine ou la déplétion de l'acide aminé méthionine augmente la production de l'isoforme Bcl-x_5 (L. Shkreta, J. Toutant et B. Chabot, données non-publiées). Cependant l'ajout d'inhibiteurs de protéasome, le bortezomib ou le MG132, contre cet effet, suggérant que l'effet de l'arrêt de la traduction est dû à la diminution de la concentration d'une protéine. De plus, les inhibiteurs de protéasome peuvent aussi annuler l'effet de l'ajout de la staurosporine ou de différentes autres agents chimiothérapeutiques qui augmentent la production de l'isoforme

pro-apoptotiques dans une variété de cellules (L. Shkreta, J. Toutant et B. Chabot, données non-publiées; Shkreta *et al.*, 2008). Ceci indique un lien possible entre ces agents apoptotiques qui pourrait être encore ce facteur limitant.

Le traitement de cellules de gliome A172 avec un oligonucléotide phosphothiorate antisense ciblant PKCα, diminuant la concentration cellulaire de cet isozyme, entraîne un arrêt de prolifération cellulaire (Dooley *et al.*, 1998). Si les cellules sont prétraitées avec le cycloheximide, il y a une très forte augmentation de la mort cellulaire. Ceci étaye bien notre hypothèse de la présence d'un facteur présent en quantité limitée et qui de plus n'est actif que lorsque phosphorylé par la voie de PKC. Le prétraitement de certains types cellulaires avec la calyculine A, un inhibiteur de la protéine phosphatase I (PP1), peut aussi annuler l'effet de l'émétine (Boon-Unge *et al.*, 2007). Il est donc possible que la déphosphorylation du facteur par PP1 puisse entraîner sa dégradation plus rapide par le protéasome.

Ainsi, en résumé, PKC pourrait phosphoryler un facteur d'épissage se liant à SB1 et qui inhibe la formation de l'isoforme Bcl-x_S. Lorsque la PKC est inactivé et que le facteur est déphosphorylé par la PP1, celui-ci pourrait être dégradé par le protéasome entraînant une formation accrue de l'isoforme pro-apoptotique Bcl-x_S. Il sera intéressant d'identifier ce facteur et de vérifier son importance dans la transduction des signaux apoptotiques de la staurosporine et des agents chimiothérapeutiques. Cette étude sera poursuivie par Alexandre Cloutier et Lulzim Shkreta.

hnRNP K réprime la production de l'isoforme pro-apoptotique Bcl-x_S

Le deuxième et dernier chapitre traite de l'implication de hnRNP K dans la répression du site d'épissage de Bcl-x_S. Lors de la délétion de régions dans l'exon 2, outre B2 et B3, nous avons aussi identifié l'élément B1, qui a été par la suite agrandi à B1AU. Cet élément est constitué de deux régions ayant des activités antagonistes : B1AC augmente l'utilisation du site x_S tandis que B1u l'inhibe (Figure 2; Revil *et al.*, 2008). De plus, B1AC peut lui-même être divisé en deux sous-éléments, B1AG et B1d, ayant chacun un effet positif. Une étude par mutations ponctuelles a identifié plusieurs sous-éléments potentiels dont la mutation réplique l'effet de la délétion des trois éléments B1AG, B1d et B1u. Ces sous-éléments sont une série de seize nucléotides dans B1AG, six nucléotides dans B1d et huit nucléotides dans B1u (Figure 3; Revil *et al.*, 2008). Étonnement, la mutation du site de liaison proposé pour RBM25 dans la région 5' de B1AG n'avait aucun effet (Zhou *et al.*, 2008). Il est possible que la mutation de deux nucléotides à la fois ne soit pas suffisante pour abolir la liaison de cette protéine. Un autre point intéressant à noter est la transition d'ESE à ESS vers les trois-quarts de B1d qui continue ensuite dans B1u, suggérant que la définition de l'activité des éléments n'est pas aussi simple que prévu. Nous avons ensuite inséré ces éléments dans l'unité d'épissage 45 constituée des sites d'épissage 5' des exons 7 et 7B de hnRNP A1 ainsi que le site 3' de l'exon « major late L2 » de l'adénovirus. Ceci transfère leur activité respective sur l'épissage alternatif de 45, quoique B1u a peu d'effet (Figure 4; Revil *et al.*, 2008). Des colonnes d'affinité avec un ARN de ces différents éléments ont identifié l'interaction de hnRNP K avec B1d et hnRNP A1 avec B1u. Pour cette dernière protéine, des essais d'épissage *in vitro* sur un transcrit de *bcl-x* démontre une augmentation de l'isoforme Bcl-x_L lors de l'ajout de hnRNP A1. Cependant, cet effet est indépendant de la présence de B1u (T. Revil et B. Chabot,

données non publiées). Une co-précipitation d'un transcrit radiomarqué de la région englobant B1AU, utilisant un anticorps contre hnRNP K, démontre la nécessité de la présence de B1 pour la liaison de cette protéine (Figure 5; Revil *et al.*, 2008). Lorsque nous utilisons un transcrit ne contenant pas l'élément B1d (ΔB1d), la co-précipitation est partiellement perdue. Ceci est encore plus marqué avec un transcrit ΔB1u. Une mutation affectant un site potentiel de hnRNP K dans B1u diminue la co-précipitation de cet ARN par hnRNP K. L'utilisation d'un anticorps contre RBM25 ne co-précipite pas d'ARN de *bcl-x*, ce qui corrèle avec l'absence d'effets des mutations du site de liaison de cette protéine. Il est possible que la liaison de cette protéine n'ait pas lieu dans nos conditions puisqu'il a été démontré que ce même anticorps pouvait co-précipiter de l'ARNm endogène. En effet, nous avons observé une région positive un peu plus en aval dont la protéine qui y lie pourrait peut-être entrer en compétition stérique avec RBM25.

Finalement, une baisse de la concentration cellulaire de hnRNP K par RNAi augmente l'utilisation du site Bcl-x_S sur les transcrits *bcl-x* endogène ou de mini-gène, dépendant de la présence de l'élément B1. Tous ces résultats indiquent l'implication de hnRNP K dans la répression du site d'épissage 5' de l'isoforme pro-apoptotique Bcl-x_S, par sa liaison avec B1, plus particulièrement B1u. Auparavant, il y avait peu d'évidences de l'implication de hnRNP K dans l'épissage alternatif (Expert-Bezancon *et al.*, 2002; Griffith *et al.*, 2006; Ule *et al.*, 2006). Un article récent démontre cependant que la déplétion de cette protéine affecte l'épissage alternatif de près de la moitié des 56 unités d'épissage vérifiées de gènes apoptotiques. Selon le pré-ARNm, hnRNP K peut augmenter ou réduire l'inclusion d'un exon, peut-être dû à l'interaction de celle-ci avec d'autres protéines (Bomsztyk *et al.*, 2004; Mikula *et al.*, 2006). Entre autres, hnRNP K peut s'associer avec Sam68 (Gorla *et al.*, 2006; Yang *et*

al., 2002), une protéine impliquée dans la régulation de l'épissage de *bcl-x* (Paronetto *et al.*, 2007). Cependant, Sam68 augmente la formation de l'isoforme Bcl-x_S tandis que hnRNP K le diminue. Une possibilité serait que hnRNP K inhibe la liaison de Sam68, peut-être dans une portion de B1AU. Cependant, nous avons effectué une diminution de la concentration de cette protéine par interférence à l'ARN sans affecter l'épissage alternatif de *bcl-x*.

La surexpression de hnRNP K est souvent observée dans les cancers (Klimek-Tomczak *et al.*, 2006; Li *et al.*, 2004a; Mandal *et al.*, 2001; Pino *et al.*, 2003). De plus, les granzymes A et K, relâchées par les cellules immunitaires, clivent plusieurs substrats cellulaires importants, dont hnRNP K (Bovenschen *et al.*, 2008). Ceci entraîne la mort des cellules infectées par des virus ou tumorales. Tel que décrit auparavant, la baisse de la concentration de cette protéine a affecté l'épissage alternatif de plusieurs gènes apoptotiques dans une étude (Venables *et al.*, 2008b). Entre autres, elle a augmenté la formation de l'isoforme pro-apoptotique de Mcl1, de la famille Bcl-2, qui est sous-représenté dans les cellules de cancer du sein (B. Chabot, données non-publiées ; Venables *et al.*, 2008a). Ces observations suggèrent encore un rôle anti-apoptotique de hnRNP K. Afin de confirmer ceci, nous avons effectué une étude par cytométrie de flux (FACS) permettant de compter les cellules mortes, apoptotiques ou vivantes. Des cellules PC-3 ont été traitées avec des concentrations croissantes de staurosporine et une diminution de la concentration de hnRNP K par interférence à l'ARN (Figure 16). Dans les cellules sans staurosporine, la déplétion de hnRNP K n'avait aucun effet sur l'apoptose. Cependant, nous observons un ralentissement de la prolifération cellulaire. L'ajout de staurosporine augmente radicalement le décompte de cellules apoptotiques, avec un pic à 250 nM dans nos conditions. Lorsqu'il y a déplétion de hnRNP K avant d'ajouter la staurosporine, ceci sensibilise davantage les cellules à l'apoptose. En effet, nous observons

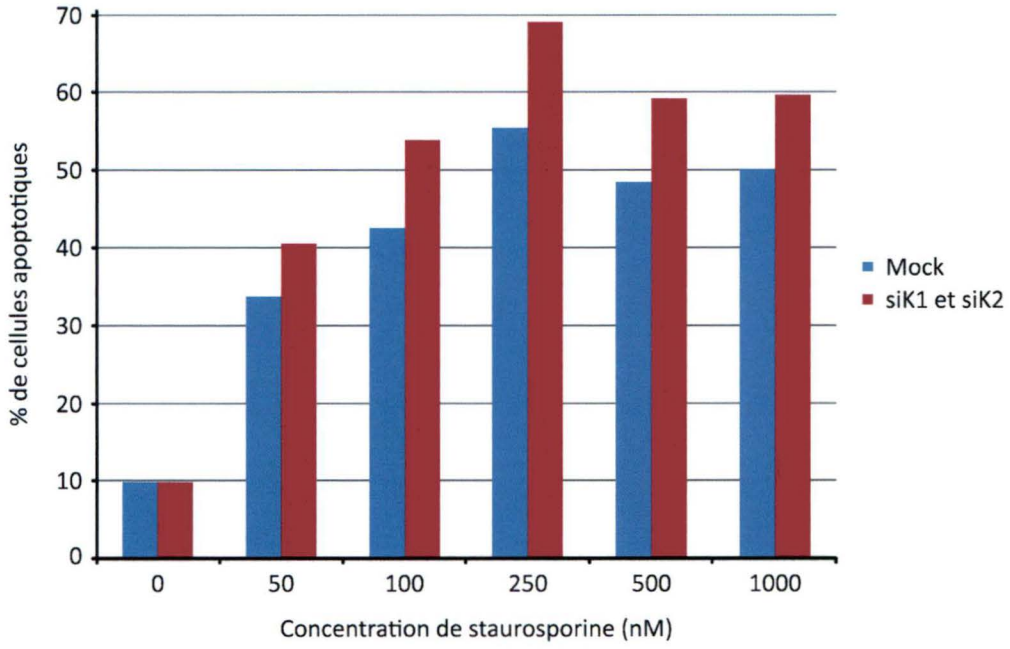

Figure 16 – La déplétion de hnRNP K sensibilise les cellules PC-3 à la staurosporine. Les cellules PC-3 ont été déplétées de hnRNP K, ou non, avec 50 nM de siK1 et 50 nM de siK2 pendant 48 heures. Par la suite, les concentrations indiquées de staurosporine ont été ajoutées. 24 heures plus tard, les cellules ont été trypsinisées et colorées à l'iodure de propidium et à l'orange acridine. Ceci permet de séparer les cellules vivantes, mortes et apoptotiques. Nous observons ici que la déplétion de hnRNP K sensibilise les cellules PC-3 à l'apoptose par la staurosporine.

en moyenne 10,4% de plus de cellules apoptotiques, et près de 14% avec 250 nM de staurosporine. Une coloration à l'annexin V, détectant la phosphatidylsérine sur la surface cellulaire des cellules apoptotiques, permet aussi de noter une très forte augmentation de l'apoptose lors de la déplétion de hnRNP K dans des cellules SKOV3 (D. Garneau, T. Revil et B. Chabot, données non-publiées).

Une étude plus approfondie de l'implication de hnRNP K dans l'apoptose est présentement effectuée par Lulzim Shkreta dans le laboratoire. Celui-ci s'intéresse notamment au fait que l'activation de p53 par la mitomycine entraîne une augmentation de l'expression de l'isoforme principal de hnRNP K, mais une diminution de l'isoforme a, dans les cellules HCT116 (Rahman-Roblick *et al.*, 2007). L'activité de l'isoforme a de hnRNP K, plus petite que l'isoforme principal, n'est pas connue. Cette augmentation serait dépendante de kinases

signalant les dommages à l'ADN ATM ou ATR (Moumen *et al.*, 2005) et inhiberait la dégradation de hnRNP K par le protéasome entraînée par l'ubiquitination due à HDM2/MDM2. hnRNP K agit aussi en tant que cofacteur de p53, augmentant la transcription de certains gènes. Il est intriguant que p53, un inducteur d'arrêt de cycle cellulaire et d'apoptose, augmente la production de hnRNP K, qui augmente les isoformes anti-apoptotiques de *bcl-x* et *Mcl1*. L'étude actuelle de hnRNP K dans l'apoptose étudie aussi les effets d'agents chimiothérapeutiques causant des bris d'ADN, et activant ainsi p53, sur l'expression de hnRNP K et l'épissage de *bcl-x*. Les résultats devraient aider à clarifier le rôle de hnRNP K dans l'épissage alternatif ainsi que son rôle dans l'apoptose en général.

Conclusions

L'étude de l'épissage alternatif de bcl-x a beaucoup avancé depuis le début de ma maîtrise en 2002. Aucune information n'était disponible à cette époque sur les mécanismes de régulation impliqués. Maintenant, douze éléments, huit facteurs d'épissage et six voies modulant l'épissage alternatif de bcl-x ont été décrits (Figure 17; Boon-Unge *et al.*, 2007; Chalfant *et al.*, 2002; Cloutier *et al.*, 2008; Garneau *et al.*, 2005; Li *et al.*, 2004b; Massiello *et al.*, 2006; Massiello *et al.*, 2004; Paronetto *et al.*, 2007; Revil *et al.*, 2008; Revil *et al.*, 2007; Zhou *et al.*, 2008).

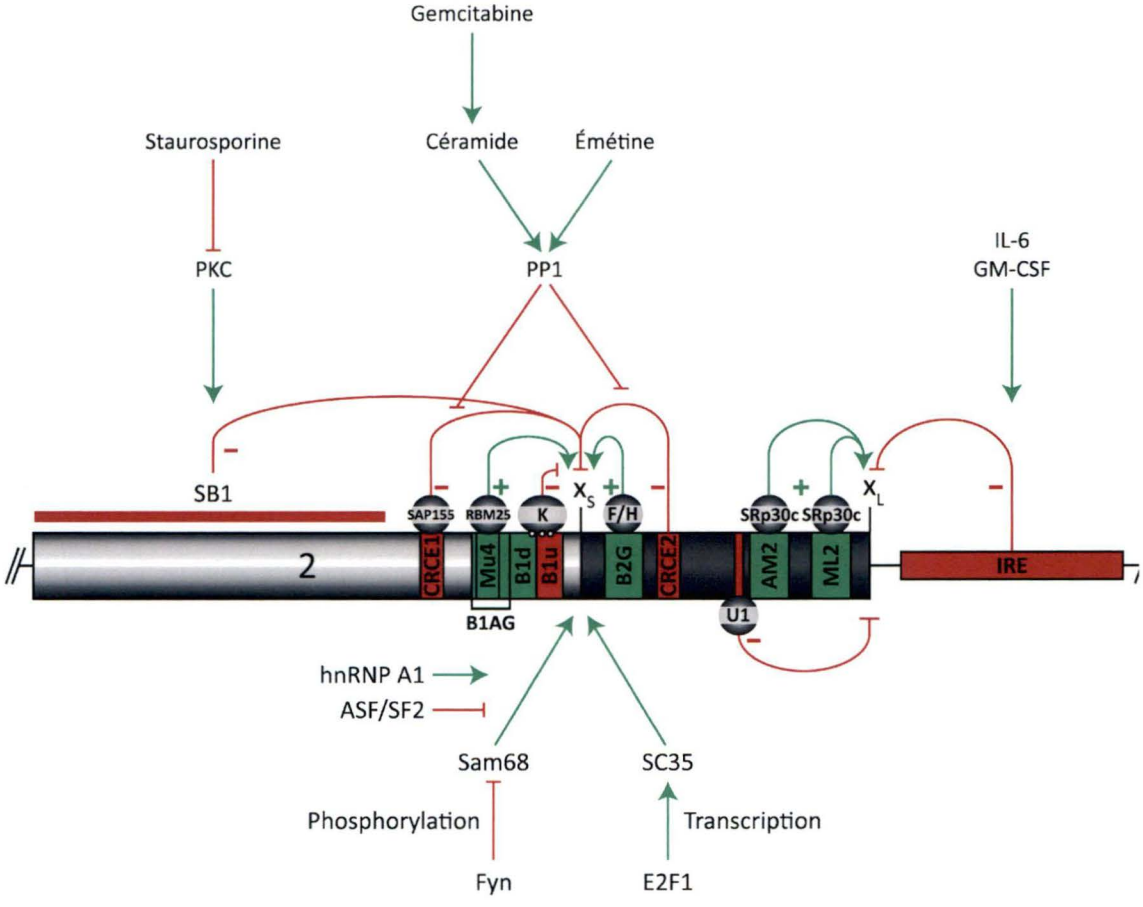

Figure 17 – La régulation de l'épissage alternatif de *bcl-x*, version fin 2008. Mes contributions à la compréhension de la régulation de l'épissage alternatif de *bcl-x*, présentées dans cette thèse, ont été ajoutées.

De celles-ci, j'ai participé à la découverte de huit éléments, quatre facteurs et une voie (Cloutier *et al.*, 2008; Garneau *et al.*, 2005; Revil *et al.*, 2008; Revil *et al.*, 2007). Malgré cette avancée, il reste encore beaucoup de pistes à poursuivre. Entre autres, il sera intéressant d'identifier le ou les facteurs liant la région SB1 ainsi que les intermédiaires entre la protéine kinase C et ceux-ci. De plus, le mécanisme d'action des éléments B1AG, B1d et B1u de la région B1AU, avec l'implication de la protéine hnRNP K, n'est pas encore complètement élucidé. Le rôle de la protéine hnRNP K dans l'apoptose sera aussi très intéressant à clarifier.

Remerciements

J'aimerais bien sûr commencer par remercier Benoit Chabot pour sa patience presqu'infinie. Ce n'est bien sûr qu'à la fin que je me rends compte de tout le temps que tu as investi dans mon éducation. Merci aussi pour cette indépendance que tu m'as laissé dans mon projet. Une phrase que tu m'as dite et que j'aime bien répéter : « C'est quand ça ne marche pas qu'on apprend. » J'ai beaucoup appris lors de cette thèse! Merci pour tout!

Merci beaucoup aux membres de mon jury de soutenance de thèse pour leur temps et leurs conseils, soit Dr Imed Gallouzi, Dr François Bachand et Dr Antonio Conconi.

À tous les membres du laboratoire, merci! Aux « gars » du 8423 : Daniel Garneau, du duo DGTR et bcl-x, Jean-François Fisette pour ses histoires farfelues, Philippe Cloutier et Caroline Paradis, l'autre gars du labo! Johanne Toutant, ma deuxième mère puisqu'elle m'a tellement aidé et menacé pour que je range ma paillasse. Et finalement merci à tous ceux qui ont ou vont travailler sur l'épissage alternatif de *bcl-x*, Luli, Laetitia, Alex, et Jordan. Aux collègues de département, dont les Orange-Outangs (ceinture en 2008!), avec qui j'ai profité de fins de semaine au chalet, gros panaches et sondes bien élimées inclus, merci aussi!

Mille mercis à mes parents pour votre intérêt et vos encouragements au cours de mes études. Sans vous, ce doctorat n'aurait été possible. Merci à Karine pour ton précieux soutien et ta compréhension dans ces dernières années de doctorat, les années les plus ardues...

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Annexe I

Garneau, D., Revil, T., Fisette, J.F., et Chabot, B. 2005. Heterogeneous nuclear ribonucleoprotein F/H proteins modulate the alternative splicing of the apoptotic mediator Bcl-x. *J Biol Chem* **280**(24): 22641-22650.

Résumé

Bcl-x est un membre de la famille Bcl-2, des régulateurs clés de l'apoptose. Le pré-ARNm de *bcl-x* est épissé alternativement pour créer Bcl-x_S et Bcl-x_L, deux isoformes associés respectivement à la favorisation et inhibition de l'apoptose. Nous avons investigué pluseurs éléments et facteurs associés à la production de ces deux variantes d'épissage. La mutation par délétion utilisant un mini-gène de *bcl-x* identifie deux régions dans l'exon 2 qui modulent la séléction des sites 5' de *bcl-x* dans les cellules HeLa. Une région (B3) est située en amont du site d'épissage x_L et augmente la production de Bcl-x_L dans les cellules et extraits d'épissage. L'autre région (B2) est située immédiatement en aval du site d'épissage x_S et favorise la production de Bcl-x_S *in vivo* et *in vitro*. Une région de 30 nucléotides riche en G (B2G) est responsable de l'activité de l'élément B2G. Nous démontrons que des protéines recombinantes hnRNP F et H lient B2G, et que la mutation de suites de G empêche la liaison. De plus, l'addition de hnRNP F à un extrait HeLa augmente la production de l'isoforme Bcl-x_S dépendant de la présence des séries de G. L'interférence à l'ARN par siRNA ciblant hnRNP F et H diminue le ratio Bcl-x_L/Bcl-x_S de transcrits de *bcl-x* issus de mini-gène ou endogène. Nos

résultats documentent un rôle positif pour les protéines hnRNP F/H dans la production du régulateur pro-apoptotique Bcl-x_S.

J'ai réalisé les expériences confirmant l'activité de l'élément et des protéines *in vivo*. La figure 1 démontre l'activité des éléments B2 et B3 dans les cellules HeLa, tandis que la figure 6 implique les protéines hnRNPs F et H dans l'activité de B2 par la déplétion à l'aide de RNAi.

hnRNP F/H Proteins Modulate the Alternative Splicing of the Apoptotic Mediator Bcl-x

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Running Title: hnRNP F/H proteins affect Bcl-x splicing

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ABSTRACT

Bcl-x is a member of the Bcl-2 family of proteins that are key regulators of apoptosis. The Bcl-x pre-mRNA is alternatively spliced to yield Bcl-x_S and Bcl-x_L, two isoforms that have been respectively associated with the promotion and the prevention of apoptosis. We have investigated some of the elements and factors involved in the production of these two splice

variants. Deletion mutagenesis using a human Bcl-x minigene identifies two regions in exon 2 that modulate Bcl-x 5' splice site selection in human HeLa cells. One region (B3) is located upstream of the Bcl-x_L 5' splice site and enforces Bcl-x_L production in cells and splicing extracts. The other region (B2) is located immediately downstream of the 5' splice site of Bcl-x_S and favors Bcl-x_S production in vivo and in vitro. A 30-nucleotide G-rich element (B2G) is responsible for the activity of the B2 element. We show that recombinant hnRNP F and H proteins bind to B2G, and mutating the G stretches abolishes binding. Moreover, the addition of hnRNP F to a HeLa extract improved the production of the Bcl-x_S variant in a manner that was dependent on the integrity of the G stretches in B2G. Consistent with the in vitro results, siRNA-mediated RNA interference targeting hnRNP F and H decreased the Bcl-x_L/Bcl-x_S ratio of plasmid-derived and endogenously produced Bcl-x transcripts. Our results document a positive role for the hnRNP F/H proteins in the production of the pro-apoptotic regulator Bcl-x_S.

INTRODUCTION

Alternative splicing is a powerful generator of proteomic diversity. It is estimated that as much as 74% of all human genes may use alternative splicing as part of their expression program (Johnson *et al.*, 2003). In the most remarkable example to date, the Drosophila DSCAM gene can potentially yield more than 38 000 different isoforms by alternative splicing (Schmucker *et al.*, 2000). Alternative splicing has the potential to alter protein activity in many important ways. In some cases, the inclusion of sequences carrying a stop codon can trigger non-sense mediated RNA decay, thereby downregulating protein expression (Lewis *et al.*, 2003; Wollerton *et al.*, 2004). In other instances, alternative splicing yields protein

variants with drastically different and sometimes antagonistic properties. This is the case with the Bcl-x pre-mRNA which experiences alternative 5' splice site utilization to produce the anti-apoptotic Bcl-x_L protein or the pro-apototic Bcl-x_S isoform (Boise *et al.*, 1993).

Bcl-x is a member of the large bcl-2 family of apoptotic genes. Bcl-x proteins modulate mitochondrial protein release, an event associated with the induction of programmed cell death. In a number of cancers and cancer cell lines, the expression of anti-apoptotic protein Bcl-x_L is increased and the ratio of the splice variants is frequently shifted to favor production of Bcl-x_L (Krajewska *et al.*, 1996; Olopade *et al.*, 1997; Reeve *et al.*, 1996; Tu *et al.*, 1998). Overexpression of Bcl-x_L is associated with decreased apoptosis in cancer cells, increased risk of metastasis, resistance to chemotherapeutic drugs and poor clinical outcome (Clarke *et al.*, 1995; Olopade *et al.*, 1997). In contrast, Bcl-x_S can induce apoptosis and sensitize cells to chemotherapeutic agents (Clarke *et al.*, 1995; Ealovega *et al.*, 1996; Mercatante *et al.*, 2001; Sumantran *et al.*, 1995; Taylor *et al.*, 1999). When cancer cells expressing high levels of Bcl-x_L are treated with an antisense oligonucleotide complementary to the 5' splice site of Bcl-x_L, splicing shifts towards the 5' splice site of Bcl-x_S and cells undergo apoptosis (Mercatante *et al.*, 2002).

Although perturbations in alternative splicing have been observed in neoplasia and metastasis (Nissim-Rafinia et Kerem, 2002; Philips et Cooper, 2000; Wu *et al.*, 2003), the identity of the factors that elicit these cancer-specific changes remains poorly documented. One study has uncovered that the progression from preneoplasia to metastasis in a mouse model of mammary tumors correlates with an increase in the abundance of specific SR

proteins, a family of proteins involved in generic and alternative splicing (Stickeler *et al.*, 1999). While changes in SR protein expression also correlate with changes in CD44 splicing (Stickeler *et al.*, 1999), alterations in SR proteins are likely to be only one of several alterations in the expression of splicing regulators during neoplasia and malignancy. For example, exclusion of the □-exon of fibroblast growth factor receptor 1 is controlled by intronic elements bound by the hnRNP I/PTB[1] (Jin *et al.*, 1999), and PTB expression is increased in malignant glioblastomas (Jin *et al.*, 2000). The situation with Bcl-x remains largely unexplored and little is known about the processes that control Bcl-x alternative splicing in normal and cancer cells. A previous study in human adenocarcinoma A549 cells indicate that ceramide can shift the ratio of Bcl-x isoforms in favor of Bcl-x_S (Chalfant *et al.*, 2002). The activity of ceramide is antagonized by protein phosphatase-1 inhibitors, which are known to alter the phosphorylated state of SR proteins (Mermoud *et al.*, 1994). Two elements flanking the Bcl-x_S 5′ splice site, a purine-rich and a pyrimidine-rich regions, are required for both basal and ceramide-induced use of the Bcl-x_S site (Massiello *et al.*, 2004). However, the direct participation of trans-acting factors in the control of Bcl-x alternative splicing remains to be documented.

We have begun to dissect the elements that control the alternative splicing of human Bcl-x. Our study identifies two elements that affect the basal Bcl-x_L/Bcl-x_S splicing ratio in human HeLa cells. Whereas the B3 element stimulates Bcl-x_L production, the B2 element activates

[1] The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; PTB, polypyrimidine tract binding protein; CMV, cytomegalovirus; siRNA, small interfering RNA.

Bcl-x_S splicing. We show that hnRNP F/H proteins mediate the activity of the B2 element in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Plasmid constructs. The human Bcl-x gene was obtained from Bacpac Resources (http://www.chori.org/bacpac/). The wild-type minigene used for in vitro splicing assays was created by the sequential PCR amplification of the 5' portion of the Bcl-x present in the BAC clone RPC1-11-243J16 using primers h1 (CTCAGAGCAACCGGGAGCTGG) and h2 (CACTGGG-CAGCTGGCAATTCT). This PCR fragment was cloned in pBluescript K+ linearized at the *Hinc*II site to generate pSB5. The 3' portion of Bcl-x was amplified from the PAC clone RPC1-5-857M17 using primers b1 (ATTATCACCAATGGAGGGTCA) and b2 (TCATTTCCGA-CTGAAGAGTGA) and the resulting fragment was cloned in pSB5 linearized at the *Eco*RV site to generate pS2.13. pX2.13 was produced by cleaving pS2.13 with *Xho*I, treating with Klenow and cutting with *Xba*I. The resulting Bcl-x fragment was then cloned into pcDNA3.1+ (Invitrogen) at the *Eco*RV and *Xba*I sites. More upstream portions of the Bcl-x gene were amplified from genomic DNA isolated from 293 cells. Amplification with primers Bx (GTCAGTCTCGAGCTTCAGAATC) and h4 (ATGCCTGATCTCTGAAGCACAG) yielded a fragment that was digested with *Hpa*I and inserted into pX2.13 at the *Hpa*I and Klenow-filled *Nhe*I site to yield pX2. pX1 was produced using the same strategy except that the upstream primer was Ex (TTATTACTCGAGGGAGGAGGAAGCAA-GCGAGG). pBcl-xΔB2 was produced by performing a PCR amplification on pS2.13 using primers Nc1 (GAGGTGATCCCCATGGC-AGCAG) and Ac1 (ATATATGTCT-ACCATTCACTACC). The PCR fragment was digested with *Nco*I

and *Acc*I and substituted for the wild type *Nco*I-*Acc*I fragment in pS2.13. pBcl-xΔB3 was constructed using the same strategy using primers Ac2 (ATATATGTAGACCGGCGGCTGGGTA) and Hi1 (TCCAAGGAGTTAACCTCTTG). The PCR fragment was digested with *Acc*I and *Hinc*II and substituted for the wild-type *Hinc*II-*Acc*I fragment of pS2.13. The pX2.13ΔB2 and pX2.13ΔB3 were produced by inserting the *Xho*I-*Xba*I fragment of the pS2.13 derivatives into pcDNA 3.1+. To generate pS2.13ΔB2G, a PCR fragment amplified from pS2.13 by primers bH11 (CGCATTGTGGCCTTT) and ds2G (AACCTCTTGTGGTGAAAT) was substituted in pS2.13ΔB2 digested with *Acc*I and *Hinc*II. pS2.13ΔB2.1 was produced from a PCR fragment amplified from pS2.13 using primers ds2.1 (CTCTCTGTCTACACCCCAGTTTAC) and NcI. The PCR fragment was substituted for the wild-type *Acc*I-*Nco*I fragment in pS2.13. pS.13ΔB2.2 was similarly produced by amplifying a fragment from pS2.13 using primers ds2.2 (CACACAGTCTACCCGAAGGAGAA) and NcI. pS2.13ΔB2□+/- were produced by isolating fragments from genomic lambda DNA obtained from an *Ava*II digestion and treated with Klenow. The DNA fragment of 73 bp (from positions 16611 to 16683 in the lambda genome) was isolated and inserted in both orientations (+ and -) into pS2.13ΔB2 digested with *Acc*I. pS2.13ΔB3s was obtained from pBluescript K+ DNA digested with *Hinf*I and treated with Klenow. The 65 bp-long DNA fragment was isolated and inserted into pS2.13ΔB3 digested with *Acc*I and treated with Klenow. Overlap PCR mutagenesis was used to generate pS-Mut1 and pS-Mut2. For pS-Mut1, two PCR reactions were performed using primers containing the mutation. A first PCR was performed with the primers mut1 (GGGATGGGGTAAACTGCCGT-CGCATTGTGGCCT) and Hi1 and a second PCR with primers Nc1 and mut1r (AGGCCACAATGCGACGGCAGT-TTACCCCATCCC). The two PCR products were used as template for a third PCR round using the Nc1 and Hi1 primers. This PCR product containing

the mutation was cut with *Nco*I and *Hinc*II and substituted for the wild-type *Nco*I-*Hinc*II fragment of pS2.13. For pS-Mut2, primers mut2 (GGGATGCCGTAAACTGCCGTCGCA-TTGTGGCCT) and mut2r (AGGCCACAATGCGACGGCAGTTT-ACGGCATCCC) were used. All plasmid constructs were extensively digested and sequenced to confirm their identity.

Culture and transfection of HeLa S3 cells. HeLa S3 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% glutamine. For transfection, 2 x 10^5 cells were plated in 35 mm^2 wells. Transfections were done using 1.5 µg of DNA and 3 µl of Lipofectamine (Invitrogen) using the manufacturer's protocol. For siRNA treatment, two successive applications of siRNAs (Integrated DNA Technologies) at 80 nM using Oligofectamine (Invitrogen) were carried out at a 24 h interval. The 20-nucleotide target sequences in hnRNP F and F/H were GCGACCGAGAACGACAUUU and GGAAGAAAUU-GUUCAGUUC, respectively. The mismatched siRNA F/Hm contained the sequence GGAGGAGAUUGUUCAGUUC. Twenty-four hours after the last siRNA application, plasmid pX2.13 was transfected using Lipofectamine (Invitrogen). RNA was extracted 24 h after the last transfection using TRIzol (Invitrogen), according to the manufacturer's protocol. The RNA was treated with DNase I for 10 min at 37°C followed by a treatment of 5 min at 70°C. One tenth of the total RNA was reverse transcribed with the OmniScript RT kit (Qiagen) at 37°C for one hour using random hexamers for monitoring the endogenous mRNAs, and BT3 (GAAGGCACAGTCGAGGCTG) for plasmid-derived mRNAs. One tenth of these reactions was used for PCR in a mixture containing 300 nM of primers, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 80 µM dNTPs and 1 unit of *Taq* polymerase. The primers used for endogenous amplification were BX3 (ATGGCAGCAGTAAAGCAAGCG) and BX2

(TCATTTCCGACTGAAGAGTGA), and BX3 and BT3 for plasmid-derived amplification. For ratio determination, the number of cpm in each band was counted directly by scanning the gel on an InstantImager (Canberra Packard). The background value was also established and subtracted from the cpm value of each band before calculating the ratio.

In vitro transcription. Templates used for in vitro transcription were made from the above plasmids by PCR amplification using PfuTurbo (Stratagene) and primers bT3 (GACCATGATTACGCCAAGCG) and bc2b (CGCTCTAGAACTAGTGGATC). PCR products were fractionated on agarose gels and purified using the QIAQuick gel extraction kit (Qiagen), according to the manufacturer's instruction. Pre-mRNAs used for in vitro splicing were synthesized using T3 RNA polymerase (USB) in the presence of cap analog and [α-^{32}P]UTP (Perkin-Elmer Life Sciences). RNA was gel-purified as described (Chabot, 1994).

In vitro splicing assays. Two fmoles of the various pre-mRNAs were incubated for 2 h in HeLa nuclear extracts (Dignam *et al.*, 1983), under standard conditions (Krainer *et al.*, 1984). Purified total RNA was resuspended in sterile water. The reverse transcription step was performed using the equivalent of 10 amol of input pre-mRNA using Omniscript (Qiagen) with the bc2b primer and was carried out at 37°C for 1 h. The reaction was followed by PCR in the presence of [α-^{32}P]dCTP (PerkinElmer Life Sciences) using the following procedure: 95°C for 3 min; 35 cycles at 93°C for 15 sec, 60°C for 30 sec (55°C for the in vivo samples), 72°C for 30 sec; and a final extension at 72°C for 3 min. Products were amplified using primers BX2 and BX3. Quantification was performed on an InstantImager, as described above.

Production of the recombinant hnRNP F/H proteins. pET15b vectors containing the hnRNP F or hnRNP H cDNAs (generously provided by Doug Black, University of California, Los Angeles) were digested using *BamH*I and *Xba*I and the cDNA fragments were cloned into pVL1392 vector. A baculovirus expression system (BD Biosciences) was used to express the recombinant proteins. The proteins were purified using Ni^{2+} affinity chromatography (Ni-NTA Agarose, Qiagen). Insect cells were pelleted by centrifugation and washed with 30 mM Tris-HCl (pH 8.0), 0.5 mM NaCl and 10% glycerol. Cells were lysed by sonication using the same buffer and the extracts were loaded onto the nickel column. The columns were washed twice with the same buffer containing 10 mM imidazole and then washed with a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 20 mM $MgCl_2$ and 5 mM ATP. Samples were eluted with 30 mM Tris-HCl (pH 8.0), 0.5 mM NaCl and 500 mM imidazole and dialyzed against buffer D (60 mM HEPES, [pH 7.9], 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 20% glycerol).

Gel-shift assays. RNA oligos were labeled at the 5' end with the T4 polynucleotide kinase (New England Biolabs). Radiolabeled oligos were incubated for 5 min at 37°C in 3.5 µl of buffer D and 2.25 µl of splicing mix (1.4 mM rATP, 9 mM $MgCl_2$, 50 mM creatine phosphate, 7% polyvinylalcohol, 5.5 mM dithiothreitol, 2U of RNAguard [Amersham Biosciences]) in the presence or the absence of 2.5 µM of hnRNP proteins. 1.75 µl of dye containing 40 µg of heparin was added before loading the samples on a 5% native acrylamide gel (29:1, acrylamide:bis-acrylamide) in Tris-glycine buffer.

Western analysis. Whole cell extracts were prepared by sonication in Laemmli sample buffer (10% glycerol, 5% □-mercaptoethanol, 2.3% SDS, 62.5 mM Tris-HCl [pH 6.8], and 0.1% bromophenol blue). Total proteins or recombinant proteins were fractionated onto a 10% polyacrylamide (29:1, acrylamide:bis-acrylamide)/SDS gel and transferred on a Hybond-C nitrocellulose membrane (Amersham Biosciences). Western blotting was performed according to standard protocols using either anti-hnRNP F or anti-hnRNP H antibodies at a dilution of 1:2000, and mixed with anti-hnRNP A1/A2 antibodies diluted 1:5000. The secondary antibody was a anti-rabbit antibody (Amersham Biosciences) diluted to 1:5000.

RESULTS

Cis-acting elements controlling 5' splice site selection in Bcl-x. As a first step towards the identification of elements involved in the control of 5' splice site selection in human Bcl-x, we tested the effect of various deletions introduced in a CMV promoter-driven Bcl-x minigene expressed in the human transformed HeLa cell line. The Bcl-x minigene X1 was constructed by combining the upstream portion of the human gene with its downstream portion. Most of intron 2, which is greater than 55 kb, was left out for convenience (Fig. 1A). However, we kept the first 1.1 kb of intron 2 because this region contains sequences that are highly conserved between human and rodents. The first 110 nucleotides of intron 2 are actually coding and are used to produce the Bcl-$x_{\square}$□□□□□□□□ (Yang *et al.*, 1997). Minigenes were transfected in HeLa cells and the relative abundance of Bcl-x_S and Bcl-x_L mRNA isoforms was estimated by RT-PCR with one of the primers corresponding to plasmid-derived sequences (Fig. 1A). In contrast to endogenous Bcl-x expression, which predominantly

produces the Bcl-x_L mRNA variant in HeLa cells (Fig. 1B, lane 2), the X1 minigene yielded considerably more of the Bcl-x_S variant (Fig. 1C, lane 2). Although the Bcl-x splicing profile from the minigene did not mimic the splicing profile of the endogenous transcripts, we nevertheless proceeded with our analysis of deletions with the goal of identifying sequences in the minigene that would alter the x_S/x_L ratio. The additional removal of exon 1 and intron 1 did not change the splicing ratio (minigene X2; Fig. 1C, lane 3). Further removing the first half of exon 2 also did not significantly alter the x_S/x_L ratio (X2.13, Fig. 1C, lane 4). In different experiments, the endogenous Bcl-x_S/Bcl-x_L ratio varied between 0.1 and 0.3, while the ratio from X2.13-derived transcripts varied between 2 and 8 (not shown). These variations in the Bcl-x splicing ratio are likely due to the status of the cells at the time of the experiment. We next tested two different exonic deletions in plasmid X2.13 (Fig. 1D). Each deletion had a strong but completely different impact on the relative abundance of the Bcl-x isoforms. The first deletion defined the B2 region, which occupies a 77-nucleotide portion downstream from the 5' splice site of Bcl-x_S (position +11 to +87 relative to the Bcl-x_S splice junction). The deletion of B2 completely abrogated the production of Bcl-x_S (Fig. 1D, lane 3), suggesting the existence of an element that is essential for the use of the 5' splice site of Bcl-x_S. In contrast, the deletion of the B3 region, which is located upstream of the 5' splice site of Bcl-x_L (position -10 to -95, relative to the Bcl-x_L splice junction), completely eliminated the production of Bcl-x_L (Fig. 1D, lane 4).

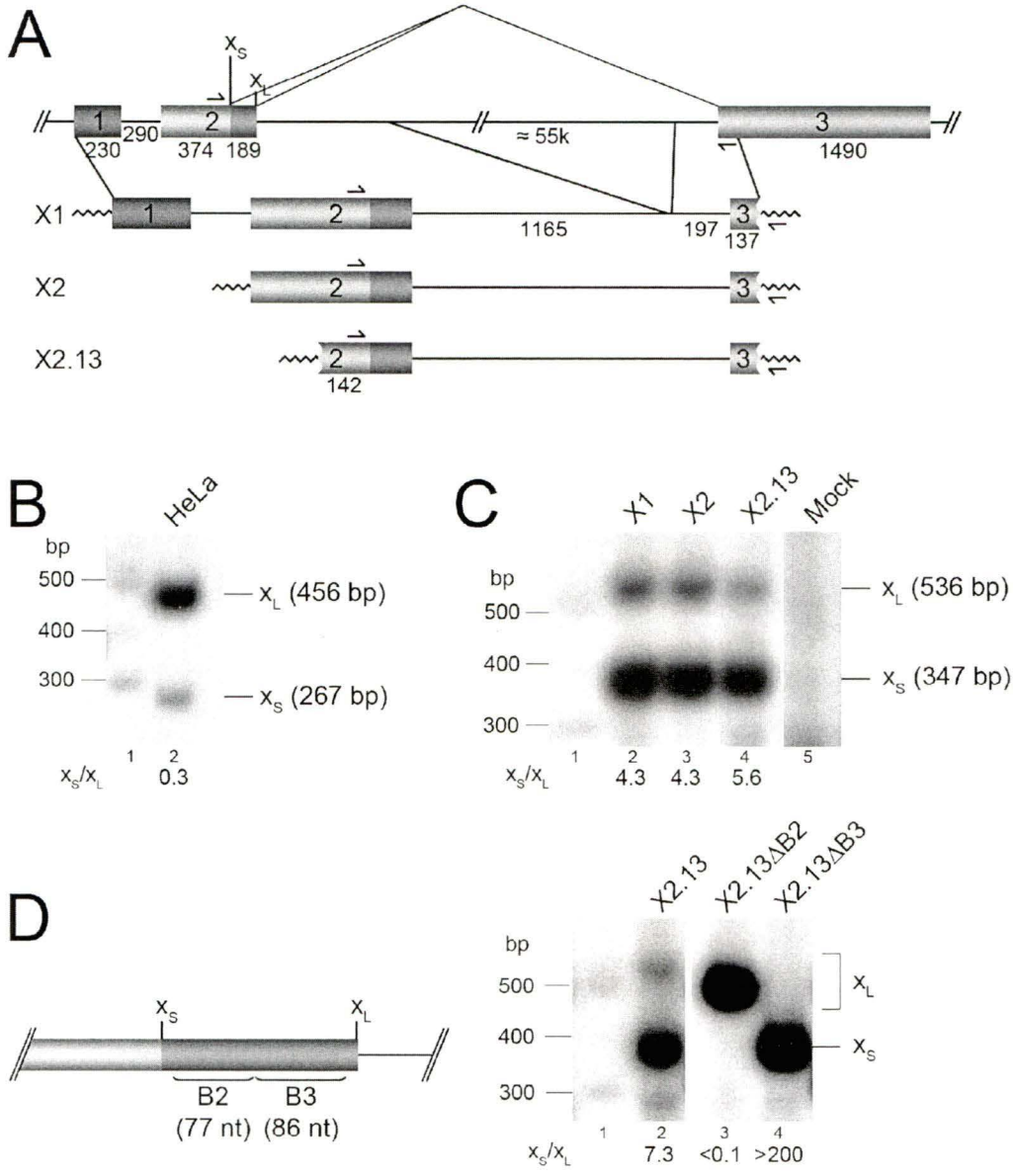

FIG. 1. Splicing of Bcl-x in HeLa cells. *A,* structure of Bcl-x and Bcl-x minigenes. The structure of the human Bcl-x gene (top) and different minigenes is drawn with exons (boxes) and introns (lines). Wavy lines indicate plasmid sequences. The size of introns and exons is indicated in nucleotides. The position of the 5' splice sites of Bcl-x_S and Bcl-x_L is indicated, as well as the position of primers used to amplify mRNA products by RT-PCR. *B,* RT-PCR assay to amplify endogenous Bcl-x mRNA products in HeLa cells. The size of the RT-PCR amplified products is indicated and the ratio of Bcl-x_S/Bcl-x_L is shown at the bottom. *C,* RT-PCR assay to amplify mRNA products expressed from transfected minigenes. The size of the amplified products corresponding to plasmid-derived Bcl-x_S and Bcl-x_L is shown, as well as the ratio between these products. *D,* RT-PCR assays on minigenes carrying deletions located in between the Bcl-x 5' splice sites. The name and size of the targeted regions are indicated on the left panel. The ratio between the Bcl-x_S and Bcl-x_L products is indicated below the lane numbers. The position of labeled DNA fragments used as markers (ladder) is indicated.

The above results point to a role for specific exonic regions in controlling the ratio of the Bcl-x isoforms. However, because the deletions change the structure of the Bcl-x_L mRNA without altering the structure of Bcl-x_S mRNA, it is possible that the deletions differentially alter the stability of the plasmid-derived Bcl-x mRNAs. To assess more directly the impact of the B2 and B3 elements on splice site selection, we carried out splicing reactions in a HeLa nuclear extract. Pre-mRNA S2.13 and derivatives were produced and incubated in splicing extracts for 2 hours. Although the efficiency of splicing for these large-intron pre-mRNAs was satisfactory (approximately 5% of input pre-mRNA), the analysis of splicing products in conventional denaturing splicing gels was complicated by the production of large lariat molecules whose migration partially overlapped when some deletions were tested (not shown). To simplify analysis we relied on performing a RT-PCR assay to selectively amplify the mRNAs produced from the synthetic pre-mRNA substrates. The amplification results indicate that pre-mRNA S2.13 was spliced to produce predominantly Bcl-x_L (Fig. 2A, lane 2). Although the Bcl-x_S/Bcl-x_L splicing ratio of pre-mRNA S2.13 varied in different experiments and in different HeLa extracts, it consistently produced more of the Bcl-x_L isoform (e.g., Fig. 2B, lane 2; Fig. 2C, lane 2, Fig. 3B, lane 2). In contrast, the B3 deletion was associated with a severe reduction in the relative abundance of the Bcl-x_L product (S2.13ΔB3; Fig. 2A, lane 4), consistent with the in vivo splicing profile of X2.13ΔB3. The B2 deletion also reproduced the in vivo effect, resulting in no detectable Bcl-x_S mRNA product (S2.13ΔB2; Fig. 2A, lane 3). To demonstrate that the 5′ splice site of Bcl-x_S remains functional in the ΔB2 construct, an antisense oligonucleotide (X5) complementary to the 5′ splice site of Bcl-x_L was added to splicing mixtures. When pre-mRNA S2.13 was incubated with increasing concentrations of

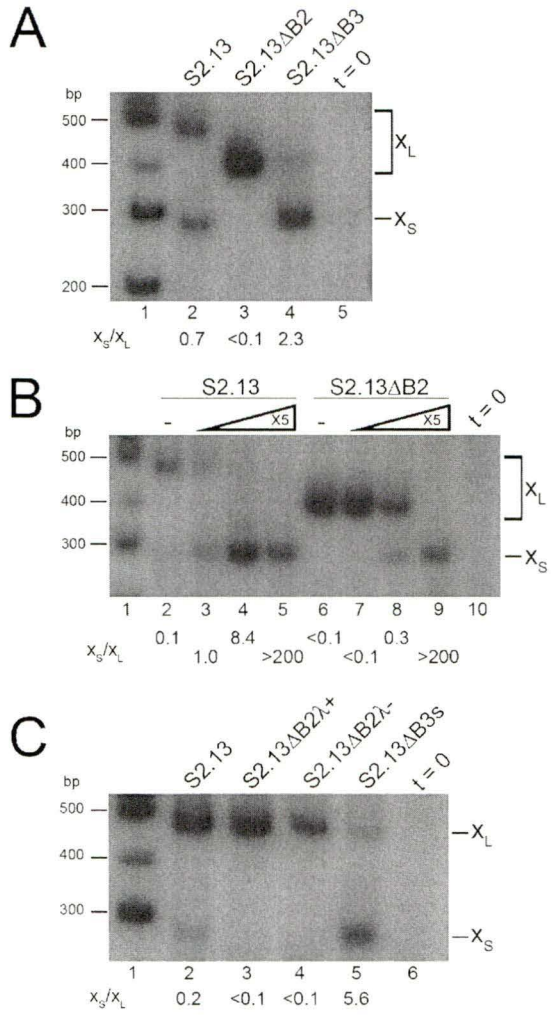

FIG. 2. Splicing of model Bcl-x pre-mRNAs in HeLa nuclear extracts. *A*, pre-mRNA S2.13 and derivatives carrying deletions in the B2 and B3 regions were tested for splicing in vitro. RT-PCR assays were carried out to monitor the relative abundance of the amplified spliced mRNA isoforms. *B*, splicing assays followed by RT-PCR amplication were performed on pre-mRNAs S2.13 and S2.13ΔB2 in the presence of the 20-nucleotide-long RNA oligo X5 (UUCUUACCCAGCCGCCGUUC, see reference Villemaire *et al.*, 2003), which is complementary to the 5' splice site of Bcl-x_L. The final concentrations of the oligonucleotide in the various splicing mixtures were 0, 8, 80 and 800 nM. *C*, pre-mRNAs carrying substitutions for the B2 and B3 elements were tested for splicing in vitro. Two sets of substitutions were tested for B2 and one for B3. Splicing was monitored by RT-PCR. In all panels, the sample corresponding to t = 0 represents an amplification performed on a splicing mixture containing pre-mRNA S2.13 that was kept on ice. The position of the Bcl-x_S and Bcl-x_L products is indicated as well as the position of labeled DNA fragments used as size markers (Ladder).

oligonucleotide X5, Bcl-x_L production decreased to favor Bcl-x_S (Fig. 2B, lanes 2-5). The X5 oligonucleotide mixed with the S2.13ΔB2 pre-mRNA also promoted the production of Bcl-x_S, although the shift was less efficient at the lowest concentrations of X5 (lanes 6-9). Thus, deleting the B2 region did not irreversibly inactivate the 5' splice site of Bcl-x_S. Moreover, the similar impact of the deletions on in vitro and in vivo Bcl-x splicing suggests that the B2 and B3 elements modulate 5' splice site selection rather than mRNA stability.

We also asked whether the effect of the deletions on alternative splicing could be caused by the juxtaposition of more distal control elements in the vicinity of the Bcl-x_S or Bcl-x_L 5' splice sites. To answer this question, we substituted the B2 and B3 elements for spacer sequences of similar length. Inserting two different spacers as replacement for B2 maintained the deficiency in Bcl-x_S production (Fig. 2C, compare S2.13 in lane 2 with S2.13ΔB2□+ and S2.13ΔB2□- in lanes 3 and 4, respectively). Likewise, substituting the B3 element for a spacer of identical length did not restore predominant Bcl-x_L production (Fig. 2C, lane 5). Thus, our results suggest that the B2 and B3 regions contain sequences that directly affect 5' splice site selection.

A subregion of B2 is bound by hnRNP F and H proteins. To investigate in more details the identity of the sequences and factors participating in the control of 5' splice site selection in Bcl-x, we focused on the 77 nucleotide-long B2 element located immediately downstream of the Bcl-x_S 5' splice site. First, we produced pre-mRNAs containing deletions of the last 22 or 48 nucleotides of B2 (S2.13ΔB2.1 and S2.13ΔB2.2, respectively; see Fig. 3A). These substrates displayed an in vitro splicing profile that indicated an impairment in Bcl-x_S

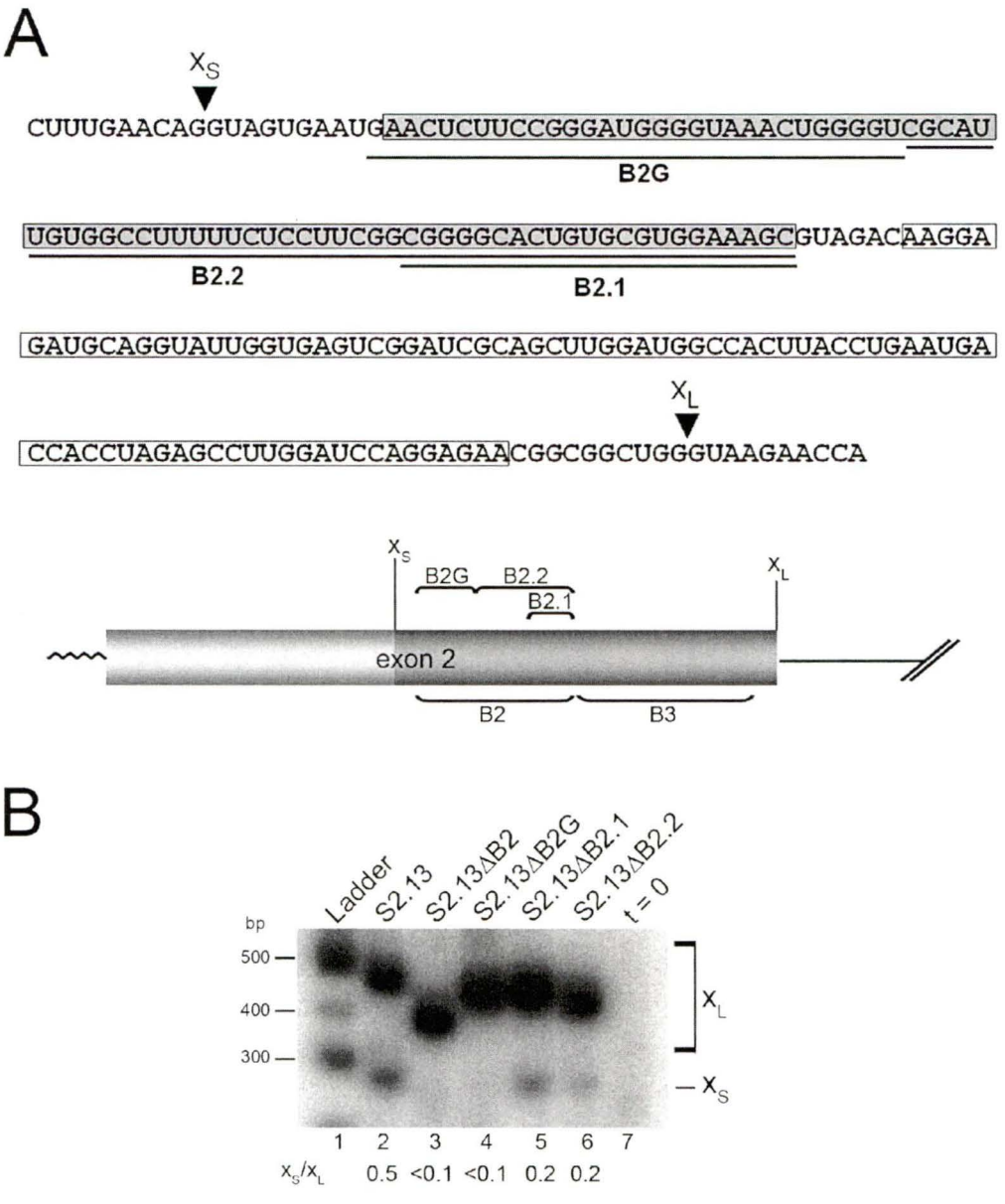

FIG 3. The first 30 nucleotides of the B2 element (B2G) enforce Bcl-x_S production. *A,* sequence of the region separating the Bcl-x_S and Bcl-x_L 5′ splice sites and structure of the deletions tested in splicing. The position of the Bcl-x 5′ splice sites is indicated. The sequences of the B2 and B3 elements are boxed (gray and white, respectively). The sequences deleted in ΔB2G, ΔB2.1 and ΔB2.2 are underlined. *B,* in vitro splicing assays of the deletion mutants. Pre-mRNAs S2.13 and derivatives were incubated in HeLa nuclear extracts for two hours. RNA was extracted and submitted to RT-PCR amplification with a specific set of primers. The position of the amplified Bcl-x_S and Bcl-x_L products is indicated and the x_S/x_L ratio of the products is shown below the lane numbers. Lane 7 (t = 0) represents an amplification performed on a splicing mixture containing pre-mRNA S2.13 that was kept on ice.

production, although the Bcl-x_S product was still detected (Fig. 3B, lanes 5 and 6). This result suggests that the 30-nucleotide portion located between positions + 10 and +39 (B2G) might make the largest contribution towards enforcing the use of the Bcl-x_S 5' splice site. Consistent with this view, a pre-mRNA substrate carrying a deletion of the B2G element yielded no Bcl-x_S products (Fig. 3B, lane 4), a splicing profile identical to S2.13ΔB2 (lane 3).

The +10 to +39 region contains the sequence CGGGAUGGGGUAAACUGGGGU (Fig. 3A). Similar G-rich sequences have been identified as signature binding sites for the hnRNP F and H proteins (Buratti *et al.*, 2004; Caputi et Zahler, 2001; Caputi et Zahler, 2002; Chen *et al.*, 1999; Chou *et al.*, 1999; Fogel et McNally, 2000; Jacquenet *et al.*, 2001; Romano *et al.*, 2002). To assess the ability of hnRNP F and H proteins to bind to this sequence, we monitored complex formation by mixing a ^{32}P-labeled RNA oligonucleotide carrying the +16 to +42 sequence (B2G oligonucleotide) with recombinant 6X-histidine-tagged hnRNP F or H produced from baculovirus-infected insect cells (Fig. 4A). As a control, we used a similarly produced His-tagged SRp30c protein (Fig. 4A, lane 3). In a gel shift assay, hnRNP F and H each retarded the migration of the B2G RNA, indicative of complex formation (Fig. 4B, lanes 21 and 22, respectively). In contrast, the baculovirus-produced His-tagged SRp30c protein did not significantly retarded the migration of the B2G RNA (lane 23). The specificity of binding by hnRNP F and H was assessed by adding increasing amounts of cold oligonucleotides. The addition of a 100-fold excess of cold B2G RNA promoted the almost complete disappearance of the labeled complexes (Fig. 4B, lanes 5 and 14), while little dissociation was observed when a similar concentration of a control RNA oligonucleotide was used (lanes 9 and 18).

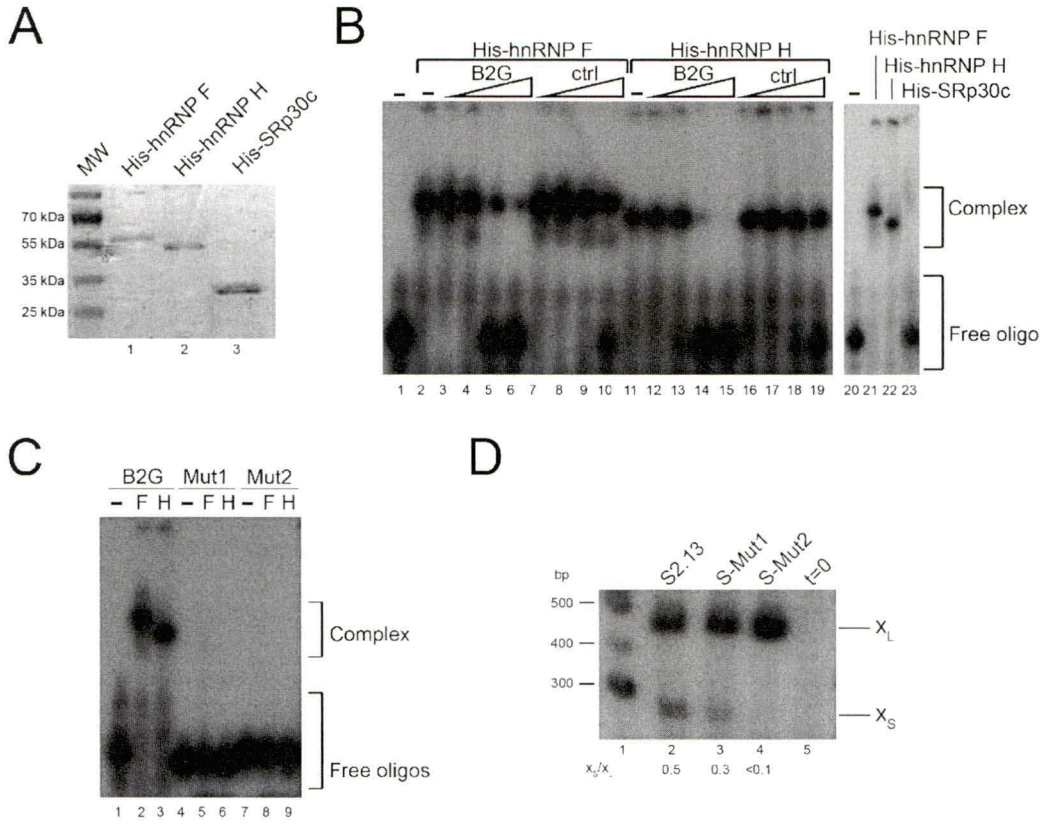

FIG. 4. G-rich portions in B2G are important for hnRNP F/H binding and the activity of the B2 element. A, his-tagged recombinant hnRNP F and H proteins as well as His-tagged SRp30c produced from baculovirus-infected cells were purified and fractionated on gels. A Coomassie-stained gel is shown and the identity of the bands as hnRNP F hnRNP H was confirmed by western analysis using anti-F and anti-H antibodies (data not shown). *B,* gel-shift assays using the recombinant hnRNP F, hnRNP H and SRp30c proteins and a labeled B2G RNA oligonucleotide (UUCCGGGAUGGGGUAAACUGGGGUCGC; positions +16 to +42 downstream of the Bcl-x_S 5' splice site). As competitors, increasing amounts of cold B2G and a control RNA oligonucleotide (ctrl: GGUAGCUGGAUUGUAGCUGCGGGUACCUUUA-GAGUAGGCC) were used corresponding to an excess of 1, 10, 100, and 500-fold relative to the labeled B2G RNA. *C,* gel-shift assays using recombinant hnRNP F and hnRNP H were carried out with B2G or RNA oligonucleotides carrying mutations (GGGG to GCCG) in the third G-rich stretch in B2G (Mut1) or the second and third G-rich stretches (Mut2). *D,* pre-mRNAs S2.13 and mutated derivatives were incubated in HeLa nuclear extract for two hours at 30°C except for the sample indicated t = 0 which was incubated for two hours on ice. Total RNA was prepared and analyzed by RT-PCR to monitor the production of the Bcl-x_S and Bcl-x_L isoforms. The position of the Bcl-x_S and Bcl-x_L amplified products is indicated as well as the quantitated Bcl-x_S/Bcl-x_L ratio.

To address the role of hnRNP F/H proteins in the activity of the B2 element, we tested the impact of mutating the G-rich stretches in the B2G portion. We produced two mutated versions: the first one (Mut1) carried a GGGG to GCCG mutation in the 3' terminal G-run, while the second one (Mut2) contained the same mutation affecting both the middle and the 3' terminal G-runs. Each mutation compromised the binding of hnRNP F and hnRNP H, as measured by gel-shift assays on RNA oligonucleotides (Fig. 4C). However, a longer exposure of the gel showed the presence of a smear above the free Mut1 RNA band incubated with hnRNP F, suggesting that mutating the last G-run in B2G did not completely eliminate hnRNP F binding (not shown). The mutations were next tested for their effect on Bcl-x splice site selection in vitro. In the context of the full B2 element, Mut1 partially reduced Bcl-x_S usage, whereas Mut2 completely abrogated it (Fig. 4D, lanes 3 and 4, respectively). Our results therefore indicate that the two quadruple G stretches in B2G are important for stable hnRNP F and H binding. In the context of the B2 element however, mutating the terminal G-run only is not sufficient to completely inactivate the element. The presence of another GGGG located 27 nt downstream of the B2G element (see Fig. 3A) may explain why mutating only one G stretch in B2G is not sufficient to completely abolish Bcl-x_S usage. Nevertheless, the fact that two intact GGGG in B2G are important both for efficient hnRNP F/H binding and Bcl-x_S splicing is consistent with the view that the hnRNP F/H proteins enforce Bcl-x_S production by binding to the G-rich elements in B2G.

hnRNP F and H stimulate splicing to the 5' splice site of Bcl-x_S in vitro. To further assess the role of hnRNP F/H proteins in Bcl-x splicing control, we tested the activity of the recombinant hnRNP F and H proteins on Bcl-x splicing in vitro. The addition of recombinant hnRNP F to a

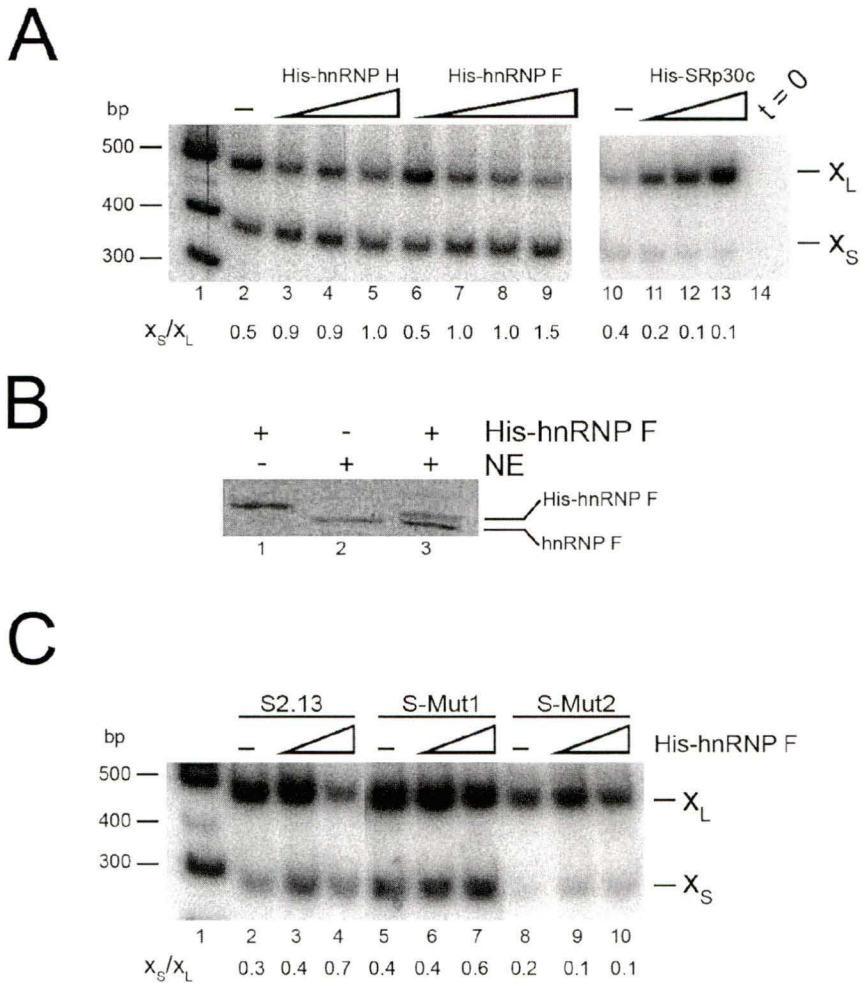

FIG. 5. hnRNP F and hnRNP H modulates the alternative splicing of Bcl-x in vitro. *A,* splicing assays in HeLa nuclear extracts were set up with pre-mRNA S2.13 in the presence of increasing amounts of His-tagged recombinant hnRNP H (0, 0.7, 1.1 and 1.5 μM), hnRNP F (0, 0.7, 1.1, 1.5 and 2.2 μM) or SRp30c (0, 0.7, 1.1 and 1.5 μM). Total RNA was isolated and amplified by RT-PCR. The position of the Bcl-x_S and Bcl-x_L products is indicated. The quantitated x_S/x_L is indicated below the lane numbers. *B,* western analysis of recombinant hnRNP F (lane 1), a HeLa nuclear extract (lane 2) and an extract supplemented with the highest amount (2.2 μM) of recombinant hnRNP F protein (lane 3). The amount of materials loaded onto the gel correspond to the amounts used in panel A, lane 8. Recombinant and endogenous hnRNP F proteins were detected using anti-hnRNP F antibodies. *C,* splicing assays using pre-mRNA S2.13, S2.13ΔB2G, S-Mut1 and S-Mut2 were carried out in the presence of increasing amounts of hnRNP F protein (0, 1.5 and 2.2 μM). The position of the Bcl-x_S and Bcl-x_L products is indicated. The x_S/x_L ratio is indicated below the lane number in panels *A* and *C*.

splicing mixture containing pre-mRNA S2.13 promoted a shift towards the production of the Bcl-x_S isoform (Fig. 5A, lanes 6-9). The largest amount of recombinant hnRNP F used to

promote this shift was equivalent to the amount of endogenous hnRNP F protein already present in the nuclear extract (Fig. 5B). Recombinant hnRNP H also improved Bcl-x_S splicing (Fig. 5A, lanes 3-5). In contrast to hnRNP F and H, the addition of His-tagged SRp30c stimulated the production of Bcl-x_L (Fig. 5A, lanes 9-12).

The splicing shift promoted by the addition of hnRNP F required a functional B2G element since adding His-tagged hnRNP F to a mixture containing the S-Mut2 pre-mRNA did not improve Bcl-x_S production (Fig, 5C, lanes 9-10). Consistent with the fact that the Mut1 mutation only weakly affected Bcl-x splicing (Fig. 4D), the addition of recombinant hnRNP F to the S-Mut1 pre-mRNA improved the production of Bcl-x_S (Fig. 5C, lanes 6-7). Overall, our results suggest that the binding of hnRNP F or hnRNP H to B2G is important for the activity of the B2 element.

hnRNP F/H knockdown reduces Bcl-x_S splicing in vivo. To address whether the hnRNP F/H proteins affect the splicing of Bcl-x in vivo, we relied on siRNA-mediated RNA interference to decrease the levels of hnRNP F and H proteins in HeLa cells. For this experiment, we used siRNAs targeting hnRNP F alone (siF) or targeting both hnRNP F and hnRNP H (siFH). Following two successive siRNA applications at a 24 h-interval and at a concentration of 80 nM, we transfected minigene X2.13. Forty-eight hours after plasmid transfection, western analysis was performed to assess the reduction in hnRNP F and H (Fig. 6A). When compared to the mock-transfected samples, protein extracts from cells that had been transfected with siF or siFH displayed a notable reduction in hnRNP F protein (Fig. 6A, lanes 1-3). siF did not affect the level of hnRNP H and the reduction in hnRNP H provoked by siFH was partial (Fig.

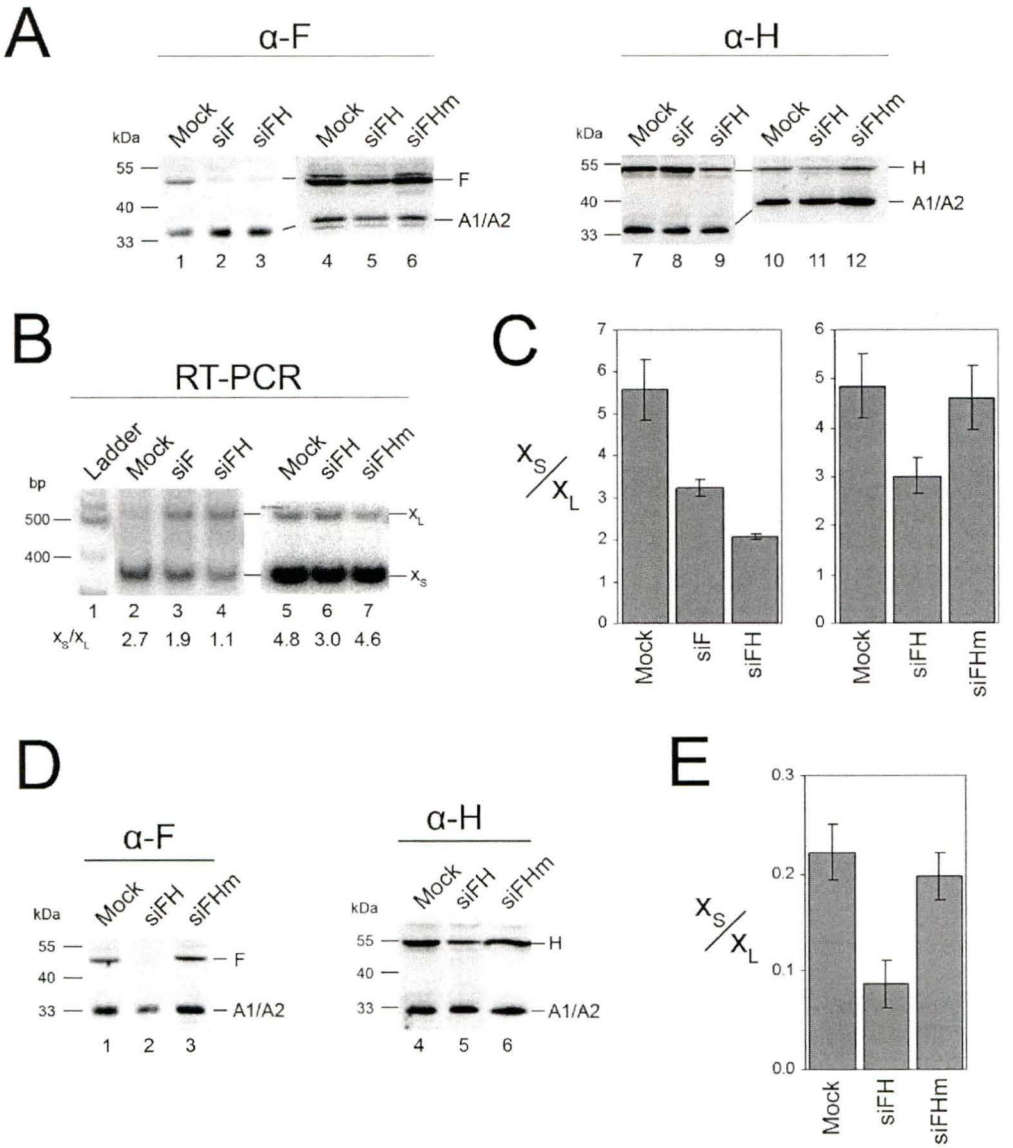

FIG. 6. hnRNP F and H proteins modulate the alternative splicing of Bcl-x splicing in vivo. In panels *A*, *B* and *C*, HeLa cells were transfected twice at a 24 h interval with siRNA mixtures, followed by transfection with the Bcl-x minigene X2.13. In panels *D* and *E*, HeLa cells were transfected with the siRNA mixtures only. Control samples were treated similarly but in the absence of siRNA (Mock). Cells were collected 72 h after the first transfection to prepare protein and RNA samples. Panels *A* and *D* represent western blots performed with antibodies specific to hnRNP F and hnRNP H (kindly provided by Doug Black). To control for total protein loading, the hnRNP A1 and A2 proteins were revealed with a polyclonal rabbit anti-A1/A2 antibody (Patry *et al.*, 2003) and the proteins comigrated in most of these gels. Panel *B* presents a RT-PCR analysis of Bcl-x transcripts derived from plasmid X2.13. The ratio of Bcl-x_S/Bcl-x_L isoform is given below the lane number. Panels *C* and *E* summarize the RT-PCR results obtained from transfections with siRNA mixtures performed in triplicate. Panel *C* monitors the Bcl-x_S/Bcl-x_L ratio on transcripts derived from plasmid X2.13, while panel *E* provides the same ratio for the endogenous Bcl-x RNAs.

6A, lanes 7-9). Specific RT-PCR assays were performed to assess the splicing of plasmid-derived Bcl-x transcripts following these treatments. The drop in hnRNP F expression was associated with a shift towards the production of the Bcl-x_L isoform (Fig. 6B, lane 3). A more important shift was observed when both hnRNP F and hnRNP H were targeted by RNA interference (lane 4). The impact of a knockdown of hnRNP F/H was confirmed by quantifying the Bcl-x_S/Bcl-x_L ratio in RNAi assays performed in triplicate (Fig. 6C, left panel). In a different experiment, we assessed the specificity of the RNAi assay by using siFHm, a version of siFH containing two mismatches with respect to the wild-type hnRNP F and H mRNAs (AGAA to GGAG). Although the impact of siFH on hnRNP F and H expression was less important in this experiment (Fig. 6A, lanes 5 and 11), a drop in Bcl-x_S was observed (Fig. 6B, lane 6). In contrast, siFHm did not reduce the level of either hnRNP F or hnRNP H (Fig. 6A, lane 6 and 12, respectively), and no shift in the Bcl-x splicing ratio was observed (Fig. 6B, lane 7). Performing the experiment in triplicate confirmed the impact and specificity of the siFH treatment (Fig. 6C, right panel).

To determine whether the knockdown of hnRNP F/K in HeLa cells also affected endogenous Bcl-x splicing, we performed RNAi assays using siFH and siFHm in the absence of a transfected plasmid. Ninety-six hours after the first application, proteins were isolated and the reduction in the abundance of hnRNP F and H proteins was confirmed by western analysis (Fig. 6D). RT-PCR amplification assays indicated that siFH elicited a shift towards Bcl-x_L, and this reduction was not seen with siFHm (Fig. 6E). Thus, the results of the RNAi assays indicate that a reduction in the cellular levels of hnRNP F and hnRNP H proteins decreases

the relative use of the x_S 5' splice site both in plasmid-derived and endogenous Bcl-x transcripts.

DISCUSSION

The regulation of Bcl-x alternative splicing is of critical importance to the apoptotic process and is highly relevant to cancer. In this study, we have identified two regions in exon 2 of Bcl-x that can modulate splicing in vitro and in vivo. The B2 and B3 elements are located in between the 5' splice sites of Bcl-x_S and Bcl-x_L, where they respectively enforce the production of the Bcl-x_S and Bcl-x_L isoforms. The activity of the B2 element is mediated predominantly by a subregion, B2G, located 10 to 39 nucleotides downstream of the Bcl-x_S donor site. Although the removal of B2G can replicate the effect of a deletion of the larger B2 element and abrogate Bcl-x_S usage, sequences downstream of B2G also appear to have some modulatory activity. A more precise mutagenesis approach targeting these sequences will be required to elucidate the exact contribution of the 3' portion of the B2 element. A recent study has identified two cis-acting elements (CRCE1 and CRCE2) that elicit the ceramide-induced switch towards Bcl-x_S in the A549 human adenocarcinoma cell line (Chalfant *et al.*, 2002). These elements also contribute to setting the basal Bcl-x_S/Bcl-x_L ratio in A549 cells. Although we have not tested the function of CRCE1 in the context of our minigene, CRCE2 was deleted when we tested the effect of sequences downstream of B2G (position + 50 to +60, relative to the 5' splice site of Bcl-x_S). In contrast to A549 cells, removing the portion that contains CRCE2 only slightly reduced the production of Bcl-x_S in a HeLa extract (S2.13ΔB2.2; Fig. 3B). The control of Bcl-x splicing therefore appears to be

complex and exon 2 may contain many control elements that allows Bcl-x splicing to respond differently to a variety of signals in distinct cellular contexts. The B3 element that we have uncovered fits in this category because deleting it severely reduced splicing to the Bcl-x_L 5' splice site. Future studies will focus on the mechanism used by B3 to control Bcl-x splicing. We have identified yet a different element in exon 2 that mediates a splicing switch in 293 cells, but not in HeLa cells, in response to the apoptotic inducer staurosporine (D. G., T. R. and B. C., unpublished data).

Notably, whereas Bcl-x_S was the predominant species produced from the minigene, endogenous Bcl-x expression in HeLa cells favors the Bcl-x_L isoform. This result indicates that the role of B2G is not as dominant in endogenously produced Bcl-x transcripts, and suggests that the minigene lacks fundamental attributes that are essential to repress Bcl-x_S and/or stimulate Bcl-x_L usage. The splicing difference between endogenous and ectopic Bcl-x transcripts can result from at least three not mutually exclusive scenarios. First, the Bcl-x gene in HeLa cells may carry mutations that affect splicing control. Second, plasmid-derived Bcl-x transcripts may be overexpressed relative to the endogenous pre-mRNAs, a situation that could lead to the sequestration of limiting regulatory molecules with an impact on the Bcl-x_S/Bcl-x_L mRNA ratio. Third, the absence of most of the ≈ 55 kb-long intron 2 sequence in the minigene may alter splicing regulation because the shortened intron lacks specific control elements. Alternatively, the large size of intron 2 suggests that commitment between the 5' splice sites of exon 2 and the 3' splice site of exon 3 takes more time to occur than for an average-length intron. Thus, a shortened intron may counteract the activity of weak control elements that normally benefit from this lag by being allowed to assemble

more productive complexes that enforce Bcl-x_L (or repress Bcl-x_S) usage. If this is the case, promoters that alter the processivity of the RNA polymerase II may have an impact on Bcl-x splice site selection. It will be interesting to examine this possibility by testing how Bcl-x is spliced when the speed of transcription is altered. Although alternative initiation sites in the mouse Bcl-x gene affect alternative splicing (Grillot *et al.*, 1997; Pecci *et al.*, 2001), it is unclear whether these changes are mediated by different transcription rates or by the presence of distinct control elements located at the 5' end of some of the pre-mRNAs.

hnRNP F/H proteins and the control of splice site selection in Bcl-x. The 30-nucleotide B2G element involved in promoting the use of the Bcl-x_S 5' splice site has the following sequence: GAACUCUUCCGGGAUGGGGUAAACUGGGGU. Mutating the last two G stretches (underlined) abrogates the activity of the B2 element. Similar motifs have been identified as binding sites for members of the hnRNP F/H/H'/2H9 family of proteins (Caputi et Zahler, 2001; Caputi et Zahler, 2002; Chen *et al.*, 1999; Chou *et al.*, 1999; Fogel et McNally, 2000; Jacquenet *et al.*, 2001; Romano *et al.*, 2002). We have shown that the recombinant hnRNP F and hnRNP H proteins can individually associate with the B2G RNA sequence, and that binding occurs in a G-stretch dependent manner. Moreover, recombinant hnRNP F and hnRNP H proteins can individually stimulate Bcl-x_S splicing in vitro and no stimulation by hnRNP F is observed when both GGGG are mutated. Most importantly, knocking down hnRNP F expression by RNA interference shifted the in vivo splicing of plasmid-derived transcript towards Bcl-x_L. A simultaneous but partial reduction in hnRNP H affected Bcl-x splicing more robustly than the knockdown of hnRNP F alone. Moreover, the combined knockdown of hnRNP F and hnRNP H expression reduced endogenous Bcl-x_S production. HnRNP F and hnRNP H may therefore

have redundant activity with regard to Bcl-x alternative splicing. However, we cannot exclude the possibility that both proteins may be simultaneously required for the activity of the B2G element. The hnRNP F and H proteins have been documented to interact with a G-rich stretch located in the intronic DCS enhancer complex that favors exon N1 inclusion in the c-*src* pre-mRNA in neuronal cells (Chou *et al.*, 1999; Min *et al.*, 1995). Whereas hnRNP H can interact with hnRNP F and is required for N1 exon splicing, hnRNP H-depleted extracts cannot be rescued by recombinant hnRNP F (Chou *et al.*, 1999). Moreover, hnRNP F does not bind strongly to the DCS RNA element (Caputi et Zahler, 2001). Given that hnRNP F and hnRNP H each bind to the Bcl-x B2G element with similar efficiency, cooperation between hnRNP F and hnRNP H may not be as critical for Bcl-x splicing control. Our future work will assess the individual or cooperative nature of hnRNP F and H-mediated control of Bcl-x splicing.

The mechanism by which hnRNP F/H proteins modulate Bcl-x splicing remains unknown. The proximity of B2G to the 5' splice site of Bcl-x_S suggests that hnRNP F/H directly stimulates the 5' splice site of Bcl-x_S. Notably, the binding of hnRNP H to an enhancer element in exon 6D of the human immunodeficiency virus pre-mRNA recruits U1 snRNP (Caputi et Zahler, 2002). If the B2G/hnRNP F/H complex directly enhances the recruitment of U1 snRNP to the 5' splice site of Bcl-x_S, this activity would be reminiscent of TIA-1 which binds very close to 5' splice sites (Del Gatto-Konczak *et al.*, 2000; Forch *et al.*, 2000; Le Guiner *et al.*, 2001), facilitates the recruitment of U1 snRNP (Forch *et al.*, 2002), and favors the production of the pro-apoptotic isoform of the Fas cell death receptor (Forch et Valcarcel, 2001).

The antagonizing activities of Bcl-x_S and Bcl-x_L in apoptosis suggest that natural variations in the levels of hnRNP F and hnRNP H proteins may influence how cells respond to apoptotic signals. The hnRNP F and H proteins display important differences in expression. Immunohistochemical analysis in normal tissues indicate that hnRNP F is often more abundant in the cytoplasm than in the nucleus, while the opposite is true for hnRNP H and the related hnRNP H' protein (Honore *et al.*, 2004). The nuclear abundance of hnRNP H is often increased in tumor tissues, such as liver carcinoma and pancreatic adenocarcinoma. HnRNP F expression appears generally high in tumors, except in liver carcinoma (Honore *et al.*, 2004). Although these observations are in appearance inconsistent with the reduced production of Bcl-x_S that would be expected in transformed tissues, the ratio between the Bcl-x_S and Bcl-x_L isoforms will likely be dictated by the coordinated contribution of several control elements and trans-acting factors. Also, post-translational modification events may modulate the activity of hnRNP F/H proteins. Although the importance of post-translational modifications in the activity of hnRNP F/H proteins has not yet been examined in details, the observation that apoptotic HL60 cells display an increased in phosphorylated hnRNP H (Navakauskiene *et al.*, 2004) is consistent with the proposed link between hnRNP F/H and apoptosis. The contribution of post-translational modifications to the activity of hnRNP F/H and Bcl-x splicing awaits further investigation.

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FOOTNOTES

We thank Stephen Hutchison, Aline Simoneau and Annie Leroux for their initial contributions into this project. We are indebted to Johanne Toutant for the preparation of nuclear extracts and the transfection assays, to Caroline Paradis for recombinant SRp30c and to Doug Black for the hnRNP F and H antibodies and cDNAs. This work was supported by a grant from the National Cancer Institute of Canada to B.C. with funds from the Canadian Cancer Society. B.C. holds a Canada Research Chair in Functional Genomics.

Annexe II

Cloutier, P., Toutant, J., Shkreta, L., Goekjian, S., Revil, T., et Chabot, B. 2008. Antagonistic Effects of the SRp30c Protein and Cryptic 5 ' Splice Sites on the Alternative Splicing of the Apoptotic Regulator Bcl-x. *J Biol Chem* **283**(31): 21315-21324.

Préambule

La sélection alternative de sites d'épissage 5' sur le pré-ARNm de *bcl-x* permet la production de deux isoformes ayant des effets antagonistes sur l'apoptose. La formation de l'isoforme pro-apoptotique Bcl-x_S est favorisée par la céramide et inhibée par la protéine kinase C à travers des éléments exoniques cis spécifiques, un d'eux lié par SAP155. Les protéines hnRNP F/H favorisent aussi la production de Bcl-x_S, ainsi que Sam68 en coopération avec hnRNP A1. Ici, nous caractérisons des éléments exoniques qui influencent l'épissage au site 5' de l'isoforme anti-apoptotique Bcl-x_L. Nous avons identifié dans une région (B3) de 86 nt située en amont du site x_L, deux éléments (ML2 et AM2) qui stimulent l'épissage au site de Bcl-x_L. SRp30c lie ces éléments et peut augmenter la formation de l'isoforme Bcl-xL de façon ML2/AM2 dépendant *in vitro* et *in vivo*. La région B3 contient aussi un élément réprimant l'utilisation de x_L. Cet élément est lié par le snRNP U1 et contient deux sites d'épissage 5' qui peuvent être utilisés quand le site d'épissage 5' de Bcl-x_L est muté ou que les éléments ML2/AM2 sont enlevés. À l'inverse, la mutation de ces deux sites d'épissage 5' cryptiques augmentent la formation de l'isoforme Bcl-x_L. Ainsi, SRp30c stimule l'épissage alternatif au

site d'épissage de Bcl-x_L, ainsi diminuant l'effet des sites répresseurs en amont liés par le snRNP U1.

Dans cet article, j'ai démontré, dans la figure 4C, que l'augmentation de l'isoforme Bcl-x_L par l'ajout de His-SRp30c dans un essai d'épissage *in vitro* n'était pas un effet non-spécifique des protéines SR. L'ajout de GST-hTRA2β n'avait pas cet effet.

Antagonistic Effects of the SRp30c Protein and Cryptic 5' Splice Sites on the Alternative Splicing of the Apoptotic Regulator Bcl-x

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Running Head: Control of Bcl-x splicing

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ABSTRACT

Alternative 5' splice site selection allows Bcl-x to produce two isoforms with opposite effects on apoptosis. The pro-apoptotic Bcl-x_S variant is upregulated by ceramide and downregulated by protein kinase C through specific *cis*-acting exonic elements, one of which is bound by SAP155. Splicing to the Bcl-x_S 5' splice site is also enforced by hnRNP F/H proteins, and by Sam68 in cooperation with hnRNP A1. Here, we have characterized exon elements that influence splicing to the 5' splice site of the anti-apoptotic Bcl-x_L isoform. Within a 86 nt region (B3) located immediately upstream of the Bcl-x_L donor site, we have identified two elements (ML2 and AM2) that stimulate splicing to the Bcl-x_L 5' splice site. SRp30c binds to these elements and can shift splicing to the 5' splice site of Bcl-x_L in an ML2/AM2-dependent manner *in vitro* and *in vivo*. The B3 region also contains an element that represses the use of Bcl-x_L. This element is bound by U1 snRNP, and contains two 5' splice sites that can be used when the Bcl-x_L 5' splice site is mutated or the ML2/AM2 elements are deleted. Conversely, mutating the cryptic 5' splice sites stimulates splicing to the Bcl-x_L site. Thus, SRp30c stimulates splicing to the downstream 5' splice site of Bcl-x_L, thereby attenuating the repressive effect of upstream U1 snRNP-binding sites.

INTRODUCTION

Alternative splicing is used by most metazoans from plants to vertebrates to expand the repertoire of proteins produced from a limited set of genes (Blencowe, 2000; Graveley, 2001). Humans in particular make ample use of this process since nearly 70% of our multi-exon genes are alternatively spliced (Johnson *et al.*, 2003).

A great diversity of factors are implicated in the control of splice site selection. In mammals, hnRNP and SR proteins form two families of RNA binding proteins that play a variety of functions in this process (Black, 2003; Martinez-Contreras *et al.*, 2006; Sanford *et al.*, 2003). For example, the binding of hnRNP A1 and hnRNP H in introns can stimulate intron definition or promote exon skipping (Chabot *et al.*, 2003; Martinez-Contreras *et al.*, 2006), a situation that may be relevant to the mechanism of action of other hnRNP-like proteins such as Nova-1 (Ule *et al.*, 2006). On the other hand, the binding of SR proteins in alternative exons often promotes exon inclusion by antagonizing the repressing activity of nearby bound hnRNP proteins (Sanford *et al.*, 2003). SR proteins can also have dual functions in splicing control. For example, while the interaction of SRp30c in the alternative exons of SMN2 and gonadotropin-release hormone stimulates its inclusion (Park *et al.*, 2006; Young *et al.*, 2002), it also associates with exonic silencer elements in tau exons 2 and 10 (Wang *et al.*, 2005) and intronic silencer element in the hnRNP A1 pre-mRNA (Paradis *et al.*, 2007; Simard et Chabot, 2002).

Although the contribution of alternative splicing to mammalian evolution, animal development and human diseases is becoming better understood (Blencowe, 2006; Faustino et Cooper, 2003; Licatalosi et Darnell, 2006; Lynch, 2004; Srebrow et Kornblihtt, 2006; Xing et Lee, 2006; Zhuo *et al.*, 2007), the functional impact of the multitude of splice variants is still poorly documented. The contribution of alternative splicing to protein function has been studied in more details in the field of apoptosis where splicing variants for receptors (e.g., Fas, LARD), adaptors (e.g., TRAF2) and several caspases display antagonistic functions (Schwerk et Schulze-Osthoff, 2005). Bcl-x remains a popular example because alternative

splicing produces a long anti-apoptotic splice variant (Bcl-x_L) and a short pro-apoptotic isoform (Bcl-x_S). The relative proportion of the Bcl-x splice isoforms is often altered in cancer cells (Krajewska *et al.*, 1996a; Krajewska *et al.*, 1996b; Olopade *et al.*, 1997; Reeve *et al.*, 1996; Tu *et al.*, 1998) and several reports suggest that the overexpression of Bcl-x_L contributes to the resistance of cancer cells to chemotherapeutic agents (Amundson *et al.*, 2000; Decaudin *et al.*, 1997).

The mechanism controlling the alternative splicing of Bcl-x has received some attention recently. In A549 cells, the production of the Bcl-x_S isoform is increased by ceramide (Chalfant *et al.*, 2002). The effect of ceramide requires PP1 (Chalfant *et al.*, 2001), a phosphatase known to act on SR proteins. Two *cis*-acting elements CRCE1 and CRCE2 that flank the Bcl-x_S 5′ splice site participate in the ceramide-mediated response (Massiello *et al.*, 2004). The U2 snRNP protein SAP155 was shown to associate with CRCE1 and its genetic depletion improved the production of Bcl-x_S (Massiello *et al.*, 2006). Cytokines also affect the ratio of the Bcl-x isoforms, and an intronic region downstream of the Bcl-x_L 5′ splice site has been implicated in mediating these effects (Li *et al.*, 2004). The phosphorylation of Sam68 by Fyn favors the production of Bcl-x_L in cooperation with hnRNP A1 (Paronetto *et al.*, 2007). Recently, we have identified an element in exon 2 (SB1) that represses splicing to the downstream Bcl-x_S 5′ splice site (Revil *et al.*, 2007). The repressor activity of SB1 requires active protein kinase C in 293 cells. Finally, we also observed that the Bcl-x_S 5′ splice site is stimulated by downstream G-rich elements bound by hnRNP F/H proteins (Garneau *et al.*, 2005). The same study also reported that deleting a 86 nt portion (B3) upstream of the 5′ splice site of Bcl-x_L decreased the production of Bcl-x_L *in vitro* and *in vivo*. Here we show that

B3 contains three elements that modulate the Bcl-x splicing ratio. Two elements stimulate the production of Bcl-x_L and their activity is mediated specifically by SRp30c. The third element contains two 5' splice site sequences and has an opposite effect on Bcl-x_L usage. Our results highlight the combinatorial nature of the B3 element and the complexity of effectors controlling the alternative splicing of Bcl-x.

EXPERIMENTAL PROCEDURES

Plasmids - pS2.13, pX2.13 and ΔB3 derivatives have been described previously (Garneau *et al.*, 2005). pS2.13ΔAM results from a religation after cleavage of pS2.13 with AccI and MscI and Klenow treatment. pS2.13ΔML was produced by amplifying with oligos HincII and XL+MscI. Products were cleaved with HincII and MscI and reintroduced in pS2.13 cut with the same enzymes. Most of the other deletion mutants were produced in a similar way: the sense oligo carrying the mutation was paired with oligo HincII while in a second reaction the antisense oligo carrying the mutation was paired with the NcoI oligonucleotide (Supplementary Table I). Both PCR products derived from these sets of oligos were mixed for a second round of amplification with the terminal oligos. The resulting fragments were cut with HincI and NcoI and ligated in pS2.13 cut with the same enzymes. The individual mutations were transferred in the pX2.13 background by purifying the XhoI (Klenow-filled)-XbaI fragments and inserting it at the EcoRV and XbaI sites of pX2.13. pVSBA and pVSBAΔB3 were constructed by amplifying the Bcl-x portion (from exon 2 to 52 bp of intron sequence) using either pX2.13 or pX2.13ΔB3 with primers XNF (GCTGGCTAGCGTTTAAACTTAAGCTTA) and XAR (TAGACGTGCAGGTGGATCTCTGACCAGAG). Plasmid pSPAd and primers AXAF (TCAG-

AGATCCACCTGCACGTCTAGG-GCGCAG) and A3R (TAATCTAGAGGCCGAGGGTTTCCGATCCAA) were used for amplification of 3' adenovirus portion that contained 77 bp and 66 bp of adenovirus intron and exon sequences, respectively. The final PCR product was amplified using the first and second PCR fragments and primers XNF and A3R. Insertion of final PCR products into NheI-blunted pcDNA3+ vector produced plasmids pVSBA and pVSBAΔB3. Primers T1187-203F (CTGTGCGTGGAAA-GCG) and A3R were used on pVSBA and pVSBA□B3 to amplify fragments that were inserted into SacII/XbaI sites of pKS, thereby providing a shortened exon 2 fragment that lacked the 5' splice site for Bcl-x_S. The SmaI-linearized plasmids pKVSBAnoS and pKVSBAΔB3noS were transcribed with T7 RNA polymerase. All plasmids were extensively digested with restriction enzymes and sequenced for confirmation. Plasmids used for the production of recombinant SRp30c have been described previously (Simard et Chabot, 2002).

Transfection assays - Plasmid transfections were carried out with polyethylenimide (Polysciences Inc., Warrington, PA, USA). Five µg of polyethylenimide were mixed with 1 µg of DNA in 200 µl of Opti-MEM I (Invitrogen, Carlsbad, CA, USA). The mixture was applied to cells for 6 hours at 37°C. DMEM supplemented with 10% of fetal bovine serum (Wisent, St-Bruno, Québec, Canada) was then added and the cells incubated for 24 to 48 hours. In all cases, RNA was extracted from cells or transfected cells using TRIzol (Invitrogen, Carlsbad, CA, USA) using the procedure described by the manufacturer. Splicing was assessed by RT-PCR. Reverse transcription was carried out with oligonucleotide RT3 and PCR was carried out with oligonucleotides X3 and RT2.

In Vitro Transcription - Pre-mRNA substrates for *in vitro* splicing assays were prepared by PCR amplification using the polymerase Pfu Turbo (Stratagene, La Jolla, CA, USA) and oligos AvT3 and X2B (0.1 µg/µl) on plasmid S2.13 and derivatives. The PCR fragments were gel-purified and transcribed using home-made T3 RNA polymerase in the presence of traces of α-^{32}P-UTP (800 Ci/mmole; PerkinElmer, Waltham, MA, USA) using standard protocols (Chabot, 1994). Transcripts were fractionated on denaturing acrylamide gels and gel-purified. Transcripts were resuspended at a final concentration of 2 fmoles/µl. In some cases, transcripts were prepared by direct transcription from the plasmid and the RNA was purified as above.

For the B3+ transcript and its derivative, PCR fragments were produced using oligos T3B3 (bearing the T3 promoter sequence) and X-5L and plasmid S2.13 or its 2xΔ derivative. Hot transcripts were produced from these templates using α-^{32}P-UTP as described in Chabot (1994). RNAs were resuspended at 100 000 cpm/µl.

In vitro Splicing - Two fmoles of transcript were incubated for 2 hours at 30°C in a mixture containing 5 µl of HeLa nuclear extract (Dignam *et al.*, 1983), 2.5 µl of polyvinylalcohol 13%, 0.5 µl ATP 12.5 mM, 0.5 µl of $MgCl_2$ 80 mM, 0.25 µl RNAguard, 0.25 µl DTT 100 mM, 0.5 µl of creatine phosphate 0.5 M and 0.5 µl of creatine kinase 2 U/µl (Roche, Mississauga, ON, Canada). After incubation, the reactions were stopped by adding 450 µl of sodium acetate 0.3 M and 0.1% SDS. Following an extraction with phenol/chloroform/isoamylalcohol, nucleic acids were precipitated with ethanol in the presence of 35 µg of glycogen. The final pellet was resuspended in 10 µl of water.

To monitor splicing, two µl of the above final RNA mixtures were reverse transcribed for one hour at 37°C with the Omniscript reverse transcriptase in a final volume of 10 µl using the Qiagen kit (Qiagen Corporation, Valencia, CA, USA), oligo X2B and RNAguard. One µl of the mixture was then incubated with 0.5 µl each of oligos X2 and X3 (0.1 µg/µl), 0.2 µl Taq DNA polymerase (5 U/µl), 2.5 µl of buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM $MgCl_2$), 20 µl of H_2O, 0.2 µl of a mixture of dNTPs at 10 mM each, and 0.1 µl of □-^{32}P [dCTP] 3000 Ci/mmole (PerkinElmer). The PCR cycle consisted in a denaturation for 3 minutes at 95°C followed by 35 cycles of 15 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C. The incubation was terminated with 3 minutes at 72°C.

Samples were fractionated on a native acrylamide gel which was exposed for 2 hours on a film with a screen. The gel was also analyzed on a PhosphorImager Storm 860 (GE Healthcare) for quantification.

Gel-Shift Assay - RNA oligos (Integrated DNA Technologies, Coralville, IA) were 5' end–labeled with polynucleotide kinase and □-^{32}P-ATP (3000 Ci/mmole; PerkinElmer), and the labeled material was purified on a MicroSpin G-25 column (GE Healthcare). Recombinant SRp30c (in D buffer) was incubated with 5000 cpm of the labeled RNAs, as in splicing mixtures in a total volume of 6.25 µl. After an incubation of 5 minutes on ice, we added 1.75 µl of a mixture containing heparine (25 µg/µl), 25% glycerol, 0.5 M EDTA and 0.2% of bromophenol blue was added. The material was loaded on a native 5% acrylamide gel (29:1 acrylamide:bisacrylamide) in 50 mM Tris, 50 mM glycine and 5% glycerol which was pre-run for 30 minutes at 130 volts. The gel was exposed on XAR film (Kodak, Rochester, NY, USA).

For complex formation analysis, native gels were run as described previously (Chabot *et al.*, 1992) and treatment with the anti-snRNA oligonucleotides were carried out as described (Gendron *et al.*, 2005).

UV Crosslinking and Immunoprecipitation Assays - Fifty thousands cpm of B3+ transcript or derivative were incubated in an *in vitro* splicing mixture (without RNAguard) for 10 minutes at 30°C. The sample was then submitted to 500 mJ of UV radiation using the UV Stratalinker 2400 (Stratagene, La Jolla, CA, USA). We then proceeded to an RNase A digestion (1 µg/µl) for 30 minutes at 37°C. Immunoprecipitation was carried out by adding 2.5 µl of anti-SRp30c antibody (Paradis *et al.*, 2007) followed with 50 µl of activated Protein A CL-4B beads (GE Healthcare) and by washing four times in a NET2 buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40, 0.5 mM DTT). All samples were resuspended in Laemmli buffer and separated by SDS-PAGE (10%). Gels were exposed 72 hours on XAR film.

Affinity chromatography - The midAM RNA oligo was coupled to agarose-adipic acid beads (Sigma, St. Louis, MO, USA) according to the manufacturer's recommendations. Twenty-five µl of these beads were then incu-bated in 93.75 µl of *in vitro* splicing mixture including the nuclear extract but without PVA for 10 minutes at 30°C. To assess the role of U1 snRNP, the nuclear extract was incubated with a 2'O-methyl oligonucleotide complementary to the 5' end of U1 snRNA. The beads were eluted twice with 200 µl of buffer D, then washed twice in 400 µl of the same buffer. The elution and washing steps were repeated with increasing amounts of KCl (0.1, 0.25, 0.5 and 1.0 M) followed by a final elution in Laemmli buffer. The eluted proteins were precipitated in trichloracetic acid and separated by SDS-PAGE. The gel

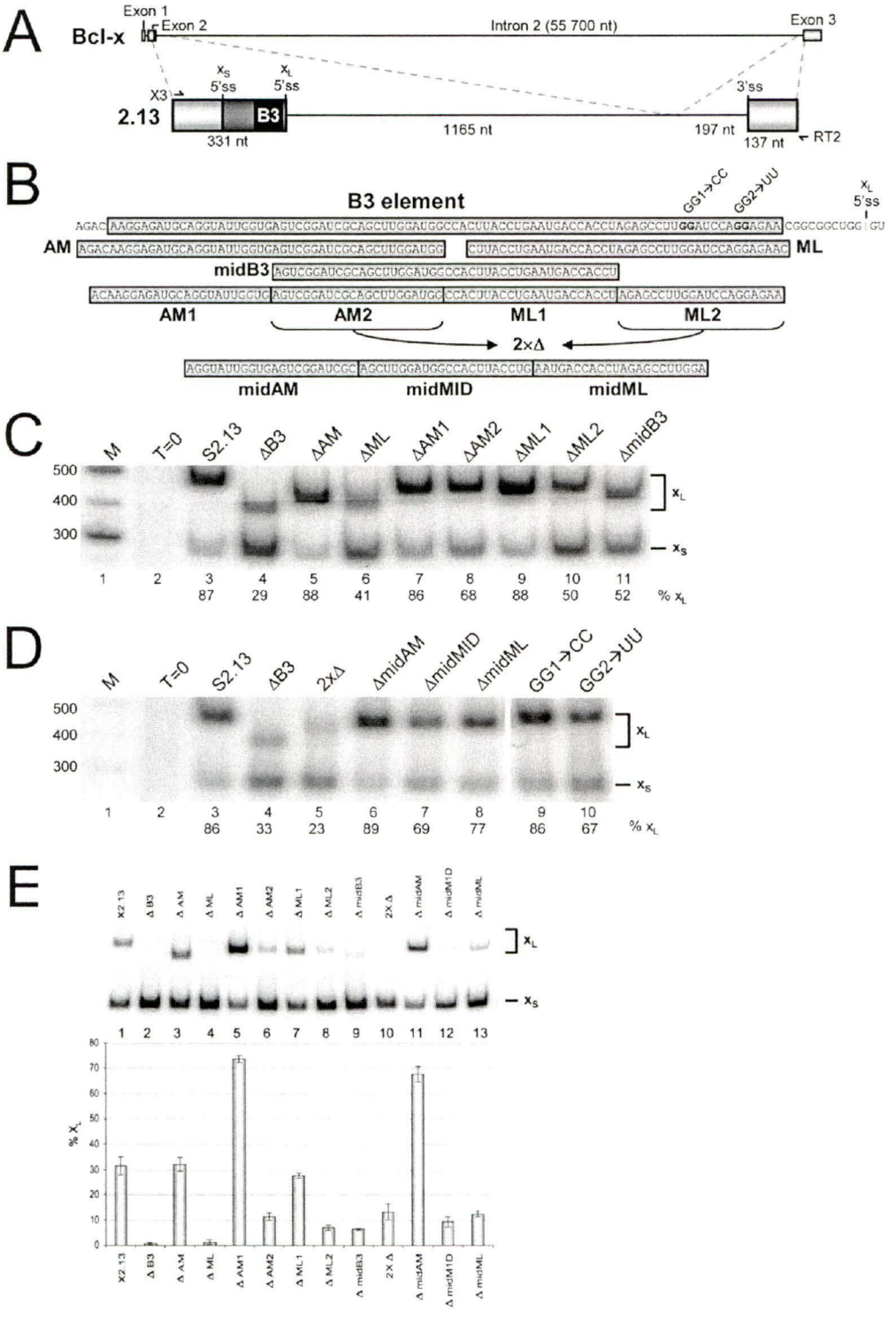

A
Bcl-x
Exon 1
Exon 2
Intron 2 (55 700 nt)
Exon 3
X3
2.13
B3
331 nt
1165 nt
197 nt
137 nt
RT2
3'ss
B
B3 element
AM
ML
midB3
AM1
AM2
ML1
ML2
2×Δ
midAM
midMID
midML
C
M
T=0
S2.13
ΔB3
ΔAM
ΔML
ΔAM1
ΔAM2
ΔML1
ΔML2
ΔmidB3
500
400
300
% x_L
D
ΔmidAM
ΔmidMID
ΔmidML
GG1→CC
GG2→UU
E
% X_L

FIG. 1. Identifying *cis*-acting elements in the B3 region of human Bcl-x exon 2. *A*, structure of the human Bcl-x gene and the 2.13 minigene. The size of the exonic and intronic portions kept in the minigene are indicated. The position of primers RT2 and X3 used to amplify exogenous transcripts is shown. *B*, illustration of the various regions of B3 that were deleted (framed) or mutated (in bold). Panels *C* and *D* show results obtained following incubation of pre-mRNA S2.13 and derivatives in HeLa splicing extracts, while panel *E* corresponds to RT-PCR performed on transfections of plasmid X2.13 and derivatives in HeLa cells. Specific RT-PCR assays were carried out to amplify Bcl-x_S and Bcl-x_L products derived from the transfected genes. The position of all products is shown. Molecular weight markers (in base pairs) are included in lane 1 of panels *C* and *D*. The percentage of Bcl-x_L amplified products is indicated below the lane numbers in panels *C* and *D*, and is represented in histograms (panel *E*) for transfections done in triplicates (with standard deviations).

was silver stained and the bands of interest were cut out and destained. In-gel trypsin digestion and LC-MS/MS analysis was performed at the Genome Quebec Innovation Center at McGill University.

RESULTS

The B3 element acts as an enhancer for the 5' splice site of Bcl-x_L - We have shown previously that the deletion of a 86 nt region upstream of the 5' splice site of Bcl-x_L decreases the use of this splice site *in vivo* and *in vitro* (Garneau *et al.*, 2005). We constructed a variety of deletion mutants (Fig. 1A and 1B) to identify regions within B3 that are responsible for this activity *in vitro* and *in vivo*. Transcripts were produced (S2.13 and derivatives) and incubated in a HeLa nuclear extract for 2 hours at 30°C. Using RT-PCR to assess the production of the x_S and x_L splice isoforms, we confirmed that B3 is important for Bcl-x_L usage in a HeLa extract since its removal shifts splicing to the Bcl-x_S 5' splice site (Fig. 1C, compare lane 4 with lane 3). Removing the 3' half of B3 (ΔML) had an effect that was almost as strong as ΔB3 (Fig. 1C, compare lane 6 with lane 4). Deleting the upstream and downstream halves of ML (ΔML1 and ΔML2, respectively) indicated that ML2 was the active portion that enforced splicing to

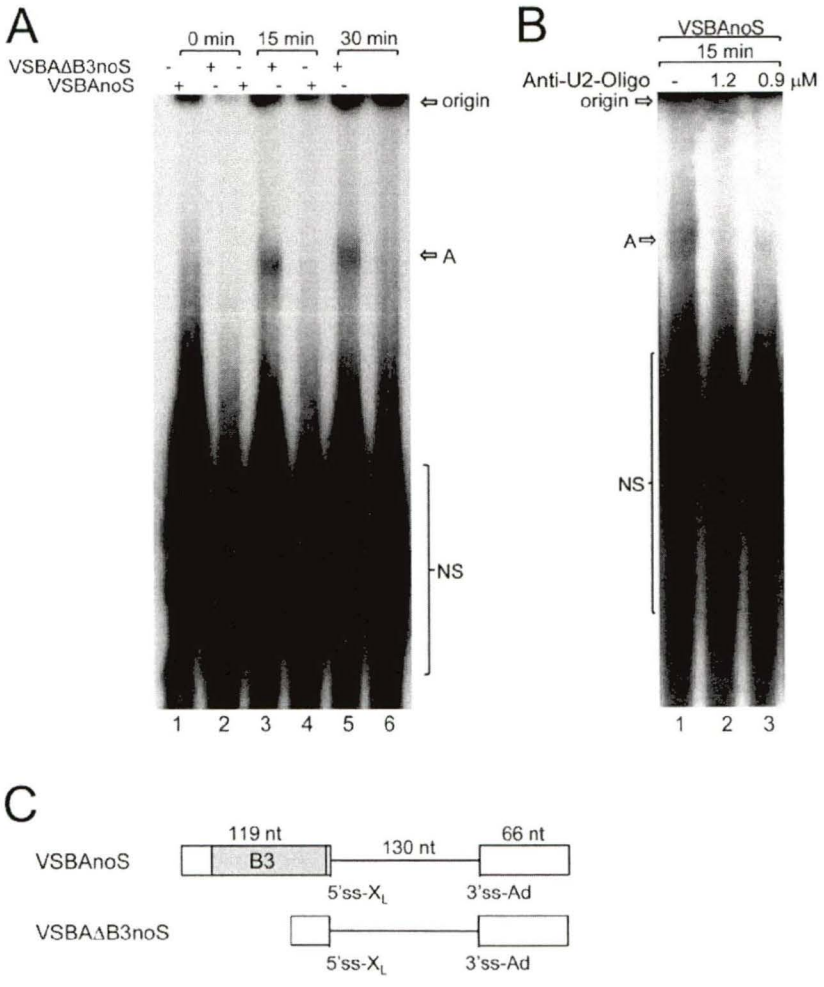


FIG. 2. B3 enhances splicing complex assembly on the 5' splice site of Bcl-x_L. *A*, a splicing complex formation assay was performed on native acrylamide gels using uniformly labeled pre-mRNAs containing or lacking the B3 element. Transcripts were incubated at 30°C in HeLa nuclear extracts and aliquots were taken at different times (0, 15 and 30 minutes). Heparine was added to a final concentration of 0.6 μg/μl. The position of non-specific (NS) complexes, complex A and the origin of the wells are indicated. *B*, to confirm the spliceosomal origin of complex A, splicing mixtures made with the B3 containing-transcript were co-incubated with a 2'OMe oligonucleotide complementary to U2 snRNA at the indicated concentrations. *C*, the structure of the transcripts used in the above assays are depicted. The size in nucleotides of different portions of the pre-mRNAs is indicated. Note that both transcripts lack the Bcl-x_S 5' splice site region.

the Bcl-x_L 5' splice site (lanes 9 and 10). In contrast, removing the first half of B3 (ΔAM) did not affect Bcl-x splicing (Fig. 1C, lane 5). However, although removing the upstream portion

of AM (ΔAM1) had no effect, removing the downstream half (ΔAM2) slightly decreased the relative level of Bcl-x_L (lane 8). A deletion encompassing AM2 and the inactive ML1 region (ΔmidB3, lane 11) confirmed the positive contribution of AM2 to Bcl-x_L splicing.

Deleting both ML2 and AM2 (2xΔ) almost completely eliminated splicing to the Bcl-x_L 5' splice site *in vitro* (Fig. 1D, lane 5). The deletion of midAM did not decrease the relative production of Bcl-x_L (Fig. 1D, lane 6), whereas ΔmidMID did (lane 7), suggesting that the active portion in AM2 is at the 3' end of this element. The most active portion of ML2 also appeared to be near its 3' end since the magnitude of the effect of ΔmidML was not as important as that observed with ΔML2 (Fig. 1D, lane 8 and Fig. 1C, lane 10, respectively).

The impact of these deletions on Bcl-x splicing was verified *in vivo* by transfecting CMV promoter-driven minigenes in HeLa cells (Fig. 1E). The parent minigene X2.13 contains the same Bcl-x portion as the S2.13 transcript used in vitro. Although transcripts produced from the parent minigene are spliced preferentially to the Bcl-x_s 5' splice site (Garneau *et al.*, 2005), the deletion of B3 abrogates the production of Bcl-x_L, an outcome also observed with ΔML and ΔML2 (Fig. 1E, lanes 2, 4 and 8). The *in vitro* impact of all the other deletions was confirmed *in vivo* except for ΔAM1 and ΔmidAM, which stimulated the use of Bcl-x_L (Fig. 1E, lanes 5 and 11) a situation not observed *in vitro* (Fig. 1C, lane 7 and Fig. 1D, lane 6) This result suggests the existence of a silencer element (see below). A silencer in the AM1-midAM region could explain why deleting AM (which removes the AM2 enhancer) had no effect (Fig. 1E, lane 3).

Thus, three regions in B3 contribute to splicing control. Although we cannot differentiate between effects on Bcl-x_L or Bcl-x_S donor sites, the argument of proximity would suggest that ML2 and the downstream portion of AM2 stimulate splicing to the Bcl-x_L 5′ splice site, whereas a sequence in midAM represses this event. To confirm the overall enhancer activity of B3 on the Bcl-x_L 5′ splice site we tested if B3 could stimulate complex formation on a simple pre-mRNA carrying the 5′ splice site of Bcl-x_L. Because complex formation is more easily observed with a strong 3′ splice site, we produced hybrid pre-mRNAs carrying the 3′ splice site of the adenovirus major late transcript. As seen in Fig. 2, deletion of B3 eliminated the U2 snRNP-dependent assembly of splicing complex A. This result therefore indicates that B3 can stimulate early spliceosome assembly on the 5′ splice site of Bcl-x_L.

A role for SRp30c in the activity of B3 - We noted that AM2 and ML2 share the sequence CUUGGAU. However, this sequence does not appear essential because mutating the GG in the ML2 site had no effect on Bcl-x splicing *in vitro* (GG1→CC; Fig. 1D, lane 9). Another feature of ML2 is that its downstream portion contains the sequence AGGAG, a sequence that we recently identified as an optimal binding site for SRp30c (Paradis *et al.*, 2007). Changing this AGGAG for AUUAG decreased splicing to the Bcl-x_L site *in vitro* (Fig. 1D, lane 10), consistent with a role for SRp30c in the activity of B3.

A potential role for SRp30c was further supported by binding assays. GST-tagged SRp30c could bind to a small RNA oligonucleotide containing the ML2 sequence and binding was compromised by the GG→UU mutation (Fig. 3A, lanes 9 and 10). Recombinant SRp30c also bound to AM2 (lane 8) even though it lacks a perfect match to AGGAG. To ask whether

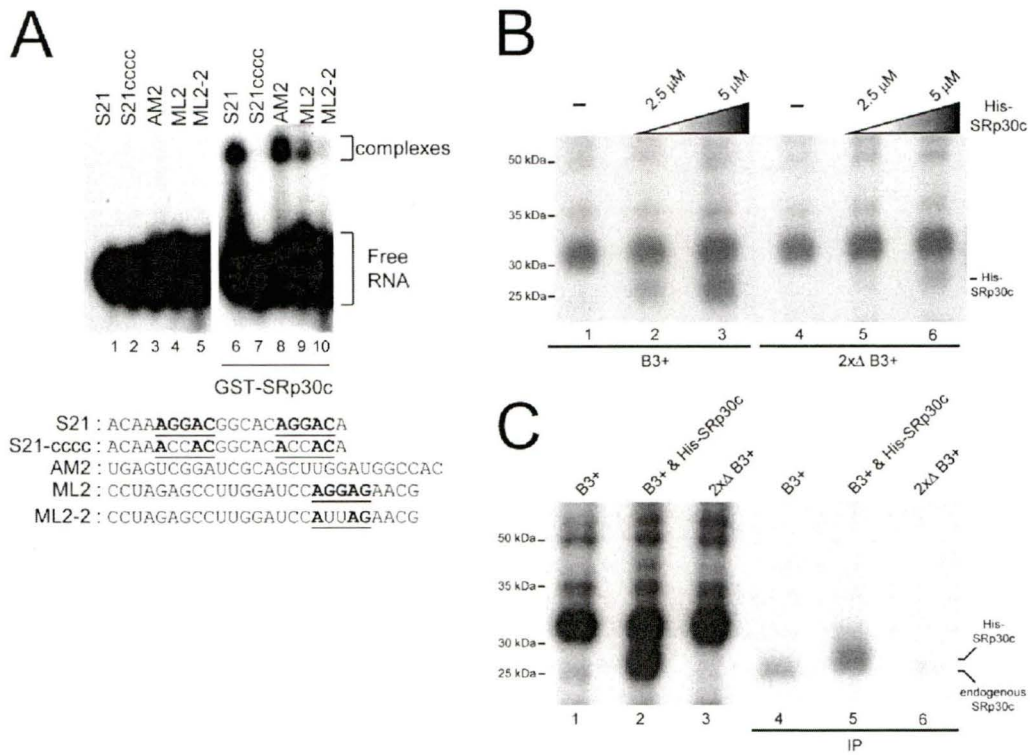


FIG. 3. SRp30c binds to B3. *A*, gel-shift assay using recombinant GST-SRp30c protein and 5'-end labeled RNA oligonucleotides containing the AM2 and ML2 elements of B3 as well as the mutated ML2-2 derivative. Oligonucleotides S21 and its mutated derivative S21-cccc act as a positive and negative control, respectively, for SRp30c binding (Paradis *et al.*, 2007). Contiguous sequences matching the high-affinity site determined by SELEX ($AGGA^{G}/_{C}$) are indicated in bold. *B*, UV crosslinking assays in nuclear extracts. Uniformly radiolabeled transcripts containing the B3 element and 20 nt of downstream sequence including the Bcl-x_L 5' splice site (B3+) or lacking the AM2 and ML2 elements (2xΔB3+) were incubated in HeLa nuclear extracts with two concentrations of His-SRp30c. The position of molecular weight markers (in kDa) and of His-SRp30c is shown. *C*, to confirm the identity of the SRp30c, UV crosslinking was followed by an immunoprecipitation step using the anti-SRp30c antibody. The position of His-SRp30c and of endogenous SRp30c is shown.

SRp30c could bind to these elements in splicing conditions, we carried out a UV crosslinking assay in a HeLa extract incubated with a uniformly labeled RNA transcript (B3+) containing the B3 element and the Bcl-x_L splice site. The crosslinking profile was compared to that of an RNA molecule lacking the AM2 and ML2 regions (2xΔB3+). Increasing amounts of recombinant His-SRp30c were added and a crosslinking product was detected with B3+ at the position expected for His-SRp30c (Fig. 3B, lane 1-3). The intensity of this band was reduced considerably when the 2xΔB3+ RNA was used and this was the only difference

noted in the crosslinking profile (Fig. 3B, lanes 4-6). The identity of this band was confirmed by performing an immunoprecipitation with anti-SRp30c antibodies on the crosslinked material (Fig. 3C, lane 5). When B3+ was incubated in an extract not supplemented with His-SRp30c, we observed a band migrating slightly faster than His-SRp30c (Fig. 2C, lane 1). This band is weaker or is absent when the assay is performed with 2xΔB3+ (lane 3). The immunoprecipitation assay indicates that a product with this behavior is recovered with the anti-SRp30c antibody (lanes 4 and 6). Thus, both recombinant and endogenous SRp30c proteins can interact with B3, and this interaction requires the AM2 and ML2 elements.

Next, we tested the role of SRp30c in the activity of B3 *in vitro*. Two concentrations (2.5 and 5.0 μM) of His-SRp30c were added to the extract. The lowest amount corresponds approximately to the amount found in 5 μl of extract (one splicing reaction) (Fig. 4B). The addition of His-SRp30c to the wild-type S2.13 pre-mRNA increased the production of Bcl-x_L (Fig. 4A, lanes 3-5), whereas it had a moderate effect when ML2 was deleted (lanes 6-8), possibly reflecting AM2-mediated activity. Indeed, removing both ML2 and AM2 nearly completely eliminated the stimulation offered by the supplementation with SRp30c (2xΔ; Fig. 4A, lanes 9-11). Notably, in this experiment, the double deletion (2xΔ) promoted the use of a cryptic 5′ splice site (x_{M1}) mapping in the midAM portion of B3 (see below). We also tested the addition of equivalent amounts of hTra2β, an SR-related protein often associated with splicing enhancer activity (Hofmann et Wirth, 2002; Nasim *et al.*, 2003; Stoilov *et al.*, 2004; Tacke *et al.*, 1998). hTra2β did not signifi cantly affect Bcl-x splicing *in vitro*, although it did stimulate the use of a distal 5′ splice site on an unrelated reporter pre-mRNA (Fig. 4C).

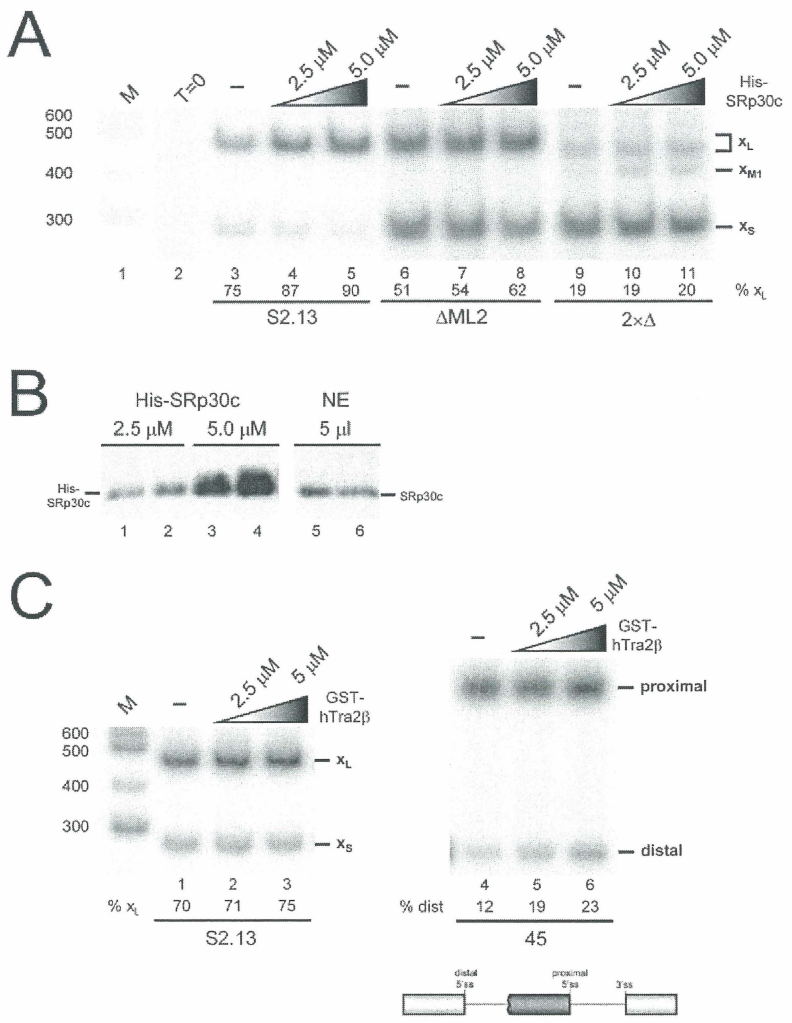


FIG. 4. SRp30c modulates the activity of B3 *in vitro*. *A*, *in vitro* splicing assays in HeLa nuclear extracts (5 μl per assay) were conducted with increasing amounts of His-SRp30c (final concentrations are indicated). Following an incubation of 2 hours, RNA was extracted and RT-PCR assays were carried out. Molecular weight markers (in base pairs) are included in lane 1 and a S2.13 transcript incubated on ice in a nuclear extract (T=0) is shown. Based on cloning and sequence analysis of the RT-PCR products, the 2xΔ pre-mRNA derivative promotes the use of a cryptic 5' splice site (x_{M1}; see text for details). *B*, western blot comparing the amount of SRp30c present in 5 μl of HeLa nuclear extract (NE) to the amounts of His-SRp30c used in the experiments shown in panel *A*. Based on this assay, we estimate that the level of SRp30c in a typical splicing reaction is approximately 2.5 μM. *C*, *in vitro* splicing assays in HeLa extracts were conducted with increasing amounts of GST-Tra2β (final concentrations are indicated) using the S2.13 Bcl-x (left) and the 45 (right) pre-mRNAs. RNA 45 contains two 5' splice sites derived from the hnRNP A1 gene competing for the adenovirus major late 3' splice site (Nasim *et al.*, 2002). RT-PCR reactions were performed as described above for S2.13 and as described previously for pre-mRNA 45 (Martinez-Contreras *et al.*, 2006).

We also tested the impact of exogenously expressing SRp30c in HeLa cells. SRp30c increased the production of the Bcl-x_L RNA isoform derived from the X2.13 minigene (Fig. 5A and 5B). No improvement in Bcl-x_L usage was detected when SRp30c was co-expressed with the variant lacking B3. The stable expression of a tagged SRp30c in HeLa and 293 cells was also associated with an increase in endogenous Bcl-x_L levels (data not shown). Thus, SRp30c can stimulate the use of the Bcl-x_L 5' splice site in a B3-dependent manner both *in vitro* and *in vivo*. We also tested the activity of the SR protein ASF/SF2, which is 74% identical to SRp30c (47). Although ASF/SF2 improved splicing to the Bcl-x_L site, consistent with previous results (Paronetto *et al.*, 2007), this effect was independent of B3 (Fig. 5C and 5D).

The silencer element contains cryptic 5' splice sites - Our deletion analysis suggested the existence of a splicing silencer in the midAM portion of B3 since removing this region increased Bcl-x_L usage *in vivo* (Fig. 1E, lane 11). With the hope of identifying a factor responsible for this activity, we carried out affinity chromatography using an RNA that contained the midAM sequence. Factors from a HeLa nuclear extract were loaded and eluted successively with 0.1, 0.25 and 0.5 and 1.0 M KCl. Compared to a column lacking RNA, approximately 15 and 7 distinct proteins were eluted at 0.25 and 0.5 M KCl, respectively (not shown). The identity of these bands is presently being investigated. Although no bands were observed in the 1.0 M KCl eluate, specific low molecular proteins remained bound to the column after the 1.0 M KCl wash (Fig. 6A, lane 6). Mass spectrometry analysis of these bands revealed that the largest one was the U1 snRNP-specific polypeptide A (7 peptides covering 43% of the protein). A faster migrating protein was B/B' (7 peptides covering 24% of the

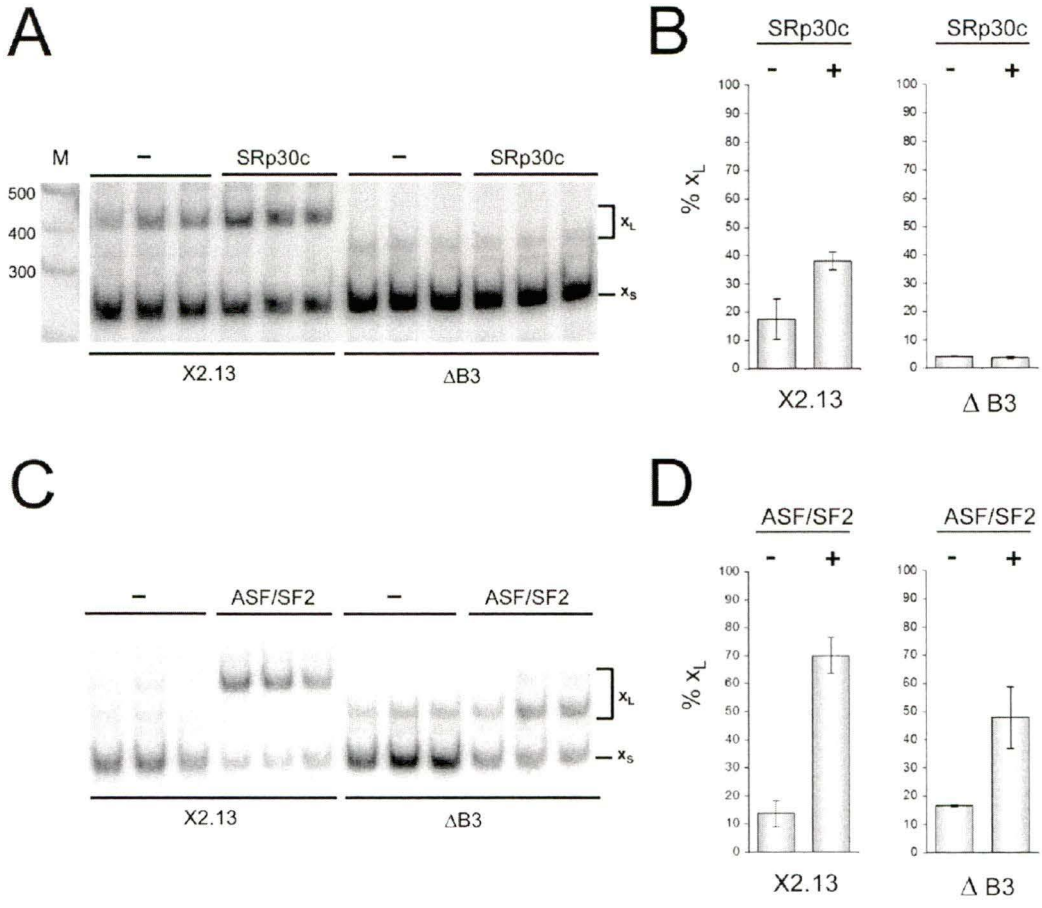
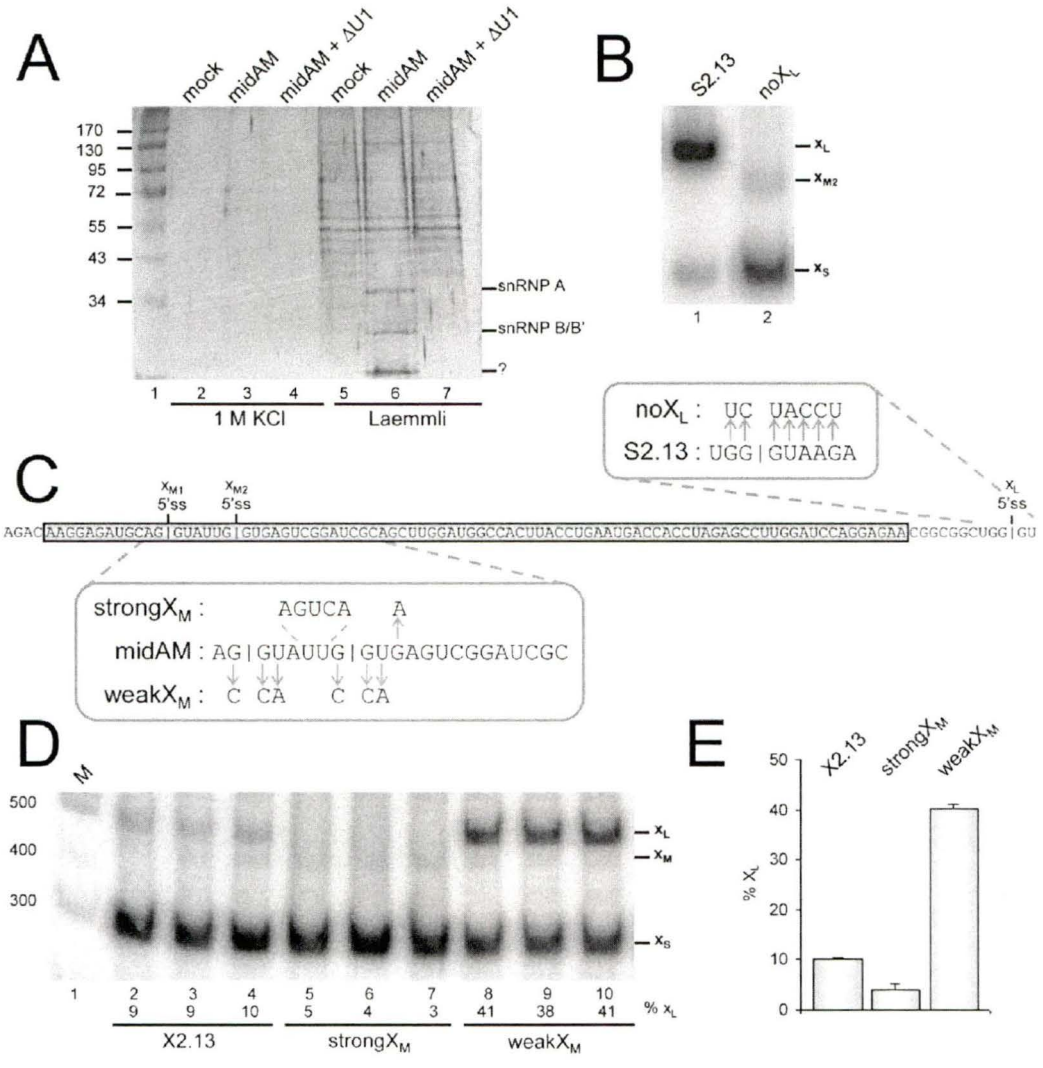


FIG. 5. Impact of overexpressing SRp30c *in vivo*. *A*, HeLa cells were transfected with a SRp30c expression vector and the X2.13 Bcl-x minigene or its ΔB3 counterpart. Triplicate transfections were processed for RT-PCR analysis. *B*, graphical representation of the results compiled from panel *A*. The change in the percentage of Bcl-x_L products elicited on X2.13 transcripts by SRp30c overexpression is highly significant ($P < 0.005$). In panels *C* and *D*, HeLa cells were transfected with a His-tagged ASF/SF2 expression vector and the X2.13 Bcl-x minigene or its ΔB3 counterpart.

protein), but the analysis of the smallest one was inconclusive even though it yielded two peptides present in U1A. When we used an extract in which the 5' end of U1 snRNA was blocked by a specific 2'O-methyl oligonucleotide, these proteins were not recovered (Fig. 6A, lane 7), suggesting that U1 snRNP binds to midAM.



F

bp score to U1

X_{M1}	CAG | GUAUUG	15
X_{M2}	UUG | GUGAGU	17
X_S	CAG | GUAGUG	16
X_L	UGG | GUAAGA	17

FIG. 6. The silencer element midAM contains pseudo 5′ splice sites. *A*, affinity chromatography using a HeLa extract was performed on beads containing an RNA oligonucleotide containing the midAM sequence. Mock-treated beads lacking RNA were also used (mock). We also used a HeLa extract that was previously incubated with a 2′O-methyl oligonucleotide complementary to the 5′ end of U1 snRNA (ΔU1). After loading, several washing steps were performed with increasing concentrations of KCl. Only the fractions eluted with 1.0 M KCl and then with Laemmli buffer are shown. The bands indicated by asterisks were cut out, destained and sent for mass spectrometry analysis. Their identity is shown when known. *B*, RT-PCR analysis of *in vitro* splicing assays performed in a HeLa extract with the S2.13 Bcl-x pre-mRNA and a derivative containing a mutated Bcl-x_L 5′ splice site (noX_L). The identity of the new PCR product (x_{M2}) was confirmed by sequencing. *C*, diagram representing the mutations used in panels *B* and *D*. Mutations to create strongX_M improve the match to the consensus 5′ splice site, while mutations in weakX_M reduce it. *D*, RT-PCR analysis of transcripts derived from the Bcl-x plasmid X2.13 and derivatives that were transfected in HeLa cells. The experiment was done in triplicate and the percentage of x_L product is shown below the lane numbers. *E*, graphical representation of the results compiled from the experiment shown in panel *D*. The differences in splicing observed with both the strongX_M and the weakX_M mutations are significant ($P < 0.005$). *F*, sequences of the Bcl-x authentic and cryptic 5′ splice sites. A base-pairing score was calculated based on the complementarity of the sequences with U1 snRNA (3 points for a GC or a CG base-pair, 2 points for AU, UA, ΨA and AΨ, and 1 point for GU, UG, GΨ and ΨG).

The binding of U1 snRNP to midAM was not totally surprising because the deletion of ML2 and AM2 (2xΔ) can promote the use of a cryptic 5′ splice site mapping in midAM (x_{M1}; Fig. 3B and Fig. 6C). Mutating the 5′ splice site of x_L (noX_L) also activated a cryptic 5′ splice site (x_{M2}) located 6 nt downstream of x_{M1} (Fig. 6B and 6C). Since we only sequenced a few clones, it is possible that both x_{M1} and x_{M2} are used in each case. These sites display relatively good complementarity to the 5′ end of U1 snRNA (Fig. 6F). To assess the role of the pseudo sites in the activity of the midAM silencer, we tested the impact of increasing or decreasing the match of theses sequences to the consensus donor site. Improving the match to the consensus of both x_M sites (Fig. 6C, strongX_M), decreased splicing to the authentic Bcl-x_L site (Fig. 6D, lane 5-7; Fig. 6E), whereas decreasing the match to the consensus (Fig. 6C, weakX_M) strongly increased the use of the Bcl-x_L 5′ splice site (Fig. 6D, lanes 8-10; Fig. 6E). Thus, our results are consistent with the view that the 5′ splice site sequences in midAM repress splicing to the downstream 5′ splice site of Bxl-x_L.

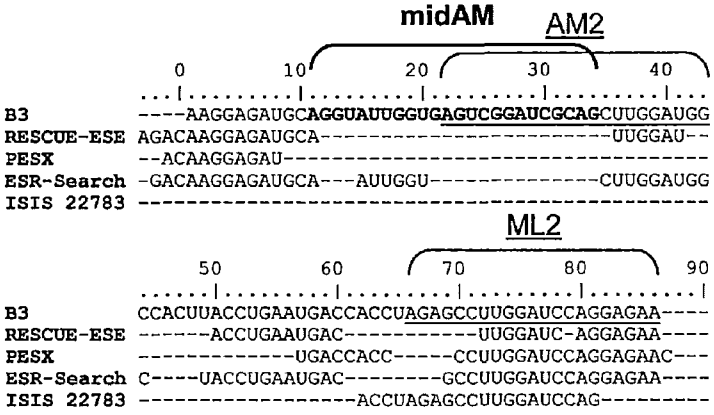


FIG. 7. Nucleotide sequence of the B3 element and alignment of enhancer sequences predicted with the search engines RESCUE-ESE, PESX and ESR-Search. Access to these engines was obtained through the web at the following URLs: RESCUE-ESE (http://genes.mit.edu/burgelab/rescue-ese/); PESX (http://cubweb.biology.columbia.edu/pesx/); ESR-Search (http://ast.bioinfo.tau.ac.il/ESR.htm). The portions corresponding to midAM, AM2 and ML2 are shown. The complementary sequence of the ISIS 22783 oligonucleotide used to inhibit the use of Bcl-x_L (Taylor *et al.*, 1999a) is also shown.

DISCUSSION

The current study links two observations made previously, specifically that the 86 nt-long B3 element is required for the optimal use of the Bcl-x_L 5' splice site, and that SRp30c can stimulate Bcl-x_L splicing *in vitro* (Garneau *et al.*, 2005). We have shown here that SRp30c can modulate the activity of B3. Two elements in B3, AM2 and ML2, individually enhanced the relative use of the Bcl-x_L 5' splice site, but the strongest effect on splicing was obtained when both ML2 and AM2 were deleted. Although we cannot rule out that B3 can repress the Bcl-x_S 5' splice site, we have shown that B3 behaves as an enhancer by helping to assemble splicing complexes on the 5' splice site of Bcl-x_L.

Consistent with an enhancer function for ML2 and AM2, search engines designed to find putative enhancer motifs (RESCUE-ESE (Fairbrother *et al.*, 2002), PESX (Zhang et Chasin,

2004), ESR-Search (Goren *et al.*, 2006)) identify such sequences in these elements (Fig. 7). The ML2 enhancer can explain the results of Taylor et al. (1999b) who used an RNA oligonucleotide complementary to an exonic portion upstream of the Bcl-x_L 5' splice site to shift splicing towards the Bcl-x_S site in cells. Retrospectively, the impact of this oligonucleotide can be explained through obstruction of a portion of the ML2 enhancer (Fig. 7).

Supplementing HeLa extracts with recombinant SRp30c stimulated Bcl-x_L splicing, and the AM2 and ML2 elements were required for this effect. This observation was confirmed *in vivo* by showing that the SRp30c-mediated stimulation in the production of Bcl-x_L was not observed when the minigene lacked B3. Recombinant SRp30c could bind to naked AM2 and ML2 RNAs. ML2 contains a sequence that fits the high-affinity binding site for SRp30c (AGGAG/C, ref. Paradis *et al.*, 2007). For AM2, it is possible that the two GGA and the GAG triplets are contributing to the binding of SRp30c. Our UV crosslinking assays indicate that SRp30c can bind to the enhancer elements in the context of a nuclear extract. SRp30c binding to these sites may then help stabilize the binding of U1 snRNP to the downstream 5' splice site of Bcl-x_L (Fig. 8).

Our characterization of B3 also identified the upstream midAM silencer. However, SRp30c also stimulated Bcl-x_L usage when the silencer element was mutated (ΔmidAM and weakX_L; data not shown), indicating that the stimulatory activity of SRp30c on the Bcl-x_L 5' splice site is probably direct and does not strictly rely on a possible repression of the upstream silencer. A direct stimulation by SRp30c is compatible with current models of action for SR proteins, which are known to facilitate U1 snRNP recruitment (Eperon *et al.*, 1993; Kohtz *et al.*, 1994;

Wu et Maniatis, 1993). More recently, the RS domain of SR proteins has also been proposed to contact directly the 5' splice site/U1 snRNA duplex to enhance binding (Shen et Green, 2006). However, it remains to be confirmed if SRp30c, which contains the shortest RS domain of the mammalian SR proteins, can stimulate the binding of U1 snRNP at the Bcl-x_L donor site.

Although increasing the levels of SRp30c in HeLa extracts and cells improved the production of Bcl-x_L, we have been unable to detect a change in the Bcl-x splicing ratio following a reduction in SRp30c levels using siRNA-mediated knockdown assays (data not shown). Although a complete knockdown may be required to observe an effect on Bcl-x splicing, this result may suggest that another factor mediates the activity of B3 in HeLa cells, a situation that would explain why endogenous SRp30c did not crosslink strongly to B3.

A role of SRp30c in Bcl-x splicing may be limited to situations that increase the level of SRp30c. For instance, SRp30c is overexpressed in activated T cells (Screaton *et al.*, 1995). The antigenic stimulation of T cells strongly predisposes cells to apoptosis and cell survival depends on the ratio of anti- and pro-apoptotic members of the Bcl-2 family (Strasser et Pellegrini, 2004). Thus, an increase in the level of SRp30c in activated T cells may facilitate the production of the anti-apoptotic Bcl-x_L isoform to help antagonize the impact of specific stimuli that would otherwise trigger apoptosis.

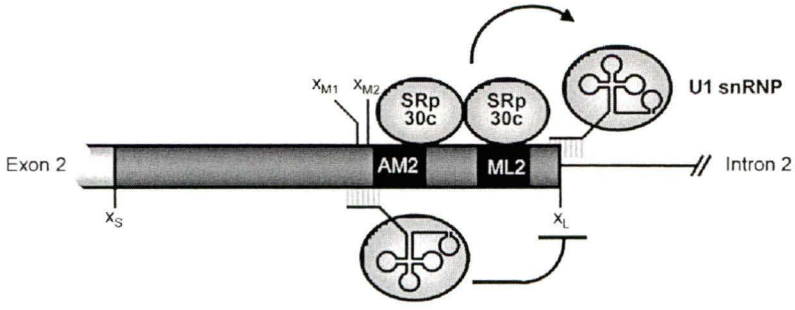


FIG. 8. Model for the activity of AM2 and ML2 elements bound by SRp30c and the pseudo donor sites. SRp30c binding stimulates the use of the Bcl-x_L 5′ splice site, possibly by stabilizing U1 snRNP at the 5′ splice site of Bcl-x_L. U1 snRNP binding to the pseudo sites is proposed to repress splicing at the Bcl-x_L 5′ splice site.

We showed that the midAM silencer element was bound by U1 snRNP, an observation consistent with the existence of two adjacent 5′ splice site sequences (CAG / GUAUUG / GUGAGU; where / indicates the splice junctions). We noted that these sites could be activated weakly when the Bcl-x_L 5′ splice site is destroyed or when the AM2 and ML2 enhancers are deleted. Why these 5′ splice site sequences are not used more efficiently is intriguing. Even improving the match of both sites such that each one was now a "perfect" 5′ splice site did not activate cryptic splicing. One possibility is that their close proximity may create strong mutual interference, as seen previously with authentic 5′ splice sites (Cunningham *et al.*, 1991).

Improving the match of the cryptic sites eliminated Bcl-x_L usage while weakening them strongly stimulated it. These results suggest that the binding of U1 snRNP to these sites can repress splicing to the authentic Bcl-x_L donor site (Fig. 8), although we cannot rule out the possibility that these mutations destroyed binding sites for other control factors. A silencer function for U1 snRNPs bound upstream of an authentic 5′ splice site is not unprecedented. Indeed, in the well-described case of the P element transposase in *Drosophila* (Siebel *et al.*,

1992), two pseudo 5' splice sites repress splicing to the authentic 5' splice site located approximately 25 nt downstream. Repression is mediated by a multicomponent complex containing U1 snRNP, PSI and hrp48 (Siebel *et al.*, 1994). Although the x_{M1} and x_{M2} junctions in Bcl-x are farther away from the downstream 5' splice site (approximately 80 nt), they are separated by 6 nt, as in transposase. It will be interesting to assess whether any of the proteins that we found interacting with midAM play a role in building a U1 snRNP-containing repressor complex.

Thus, B3 contains enhancer and silencer activities, but the dominant impact is that of an enhancer. This enhancement can be mediated by SRp30c and appears to counteract the repressive activity of upstream U1 snRNP binding sites. It is intriguing to speculate that this organization may act as a checkpoint that helps the cell gauges the ability of its splicing machinery to distinguish authentic from aberrant 5' splice sites. A general reduction in this discriminatory ability would decrease the production of Bcl-x_L, thereby predisposing cells to apoptosis.

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FOOTNOTES

*We thank Ian Eperon for Tra2, Daniel Garneau and Caroline Paradis for reagents, and Catherine Desrosiers for the Taq and Pfu polymerases. We thank Jean-François Fisette for his assistance. This study was supported by the Canadian Institute of Health Research. B.C. holds a Canada Research Chair in Functional Genomics.

The abbreviations used are: snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein; hnRNP, heterogenous nuclear ribonucleoprotein.